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Ollscoil na hÉireann, Corcaigh

National University of Ireland, Cork

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Mining the Microbiome for Markers of Microbiota-Gut Brain Communication and Mental Health

Thesis presented by

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For the degree of

Doctor of Philosophy

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1.1 Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

1.2 Author Contribution

I strive to be a bioinformatician. As such, all non-bioinformatic work, including sample preparation, collection, the sequencing of samples and all other *wet lab* work, was performed by my co-authors and the manuscripts presented here would not have been possible without them. All microbiome, metagenomics and metabolomics bioinformatics work conducted in this thesis was performed independently by the author with the following exceptions:

In chapters 3.1, 3.2, 3.3 and 3.4 the 16S sequencing data was aggregated to the .biom format by Dr. Fiona Fouhy. In chapter 4.4 it was done by Dr. Orla O’Sullivan.

In chapter 3.4, the whole genome shotgun sequencing data was annotated and aggregated to the count table format by Dr. Veronica L. Peterson.

In all cases, the rest of the bioinformatics analysis was performed by me.

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I am grateful to, as Sofia put it so aptly, the saints of the lab. Gerry, Anna, Kieran and later Ken have always been the first responders, whether I was shooting holes through dodgy data sets, struggling with the design of a study, wanted to brainstorm on ambitious and far-fetched ideas, had lost access to my email account or was just in the mood for an evening chat, you were always ready. Thank you.

I would also like to thank my friends and colleagues in the Cryan & Dinan lab as well as in the Claesson lab, past and present. You have been wonderful company and I

look forward to our further collaborations. I have gained many new friends from among you and for this I am grateful. In particular, I would like to acknowledge and thank all my colleagues who did the hard work of collecting, preparing and analysing samples in the wetlab. Bioinformaticians need real data and my work would have been completely impossible were it not for your hard work.

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I am especially grateful to my amazing wife Anne, who, to live with me in Cork, moved countries while heavily pregnant. Despite things not always being easy for us, we have always been a strong team and we have not faltered. I would also like to thank my son Trystan for teaching us the value of sleep, we will never take it for granted again. I look back fondly at our time so far and I can't wait to find out what the future has in store for us.

I dedicate this thesis to my grandfather Einar Clausen in particular, who was a microbiologist at the university of Tromsø. I am grateful to him for encouraging my fascination with biology by giving me a microscope for my birthday and for sparking my interest in mathematics by helping me discover the properties of π .

1.4 Abstract

There has been a growing acknowledgement of the involvement of the gut microbiome - the collection of microbes that reside in our gut - in regulating our mood and behaviour. This phenomenon is referred to as the microbiota-gut-brain axis. While our techniques to measure the presence and abundance of these microbes has been steadily improving, there are many factors that prevent us from understanding what aspects of the gut microbiome specifically influence the microbiota-gut-brain axis. In this thesis, we set out to identify and investigate aspects of the microbiome that are informative to gut-brain communication. We do this by investigating the state of the gut microbiome in both health and disease, as well as after supplementing or perturbing it.

While all of the work presented here is based on real data from real experiments, the thesis has a strong bioinformatics focus, that means that while the physiological background and interpretation are important, my role in these projects has been to bioinformatically and statistically zoom in on the features of the microbiome that are the most informative to our questions. As such, all results will be discussed from a primarily bioinformatics point of view.

Two main aspects of the gut microbiome came out as the most promising features to measure, namely functional capacity and volatility. Traditionally, the microbiome is thought of as a collection of microbes and most analysis is done on the taxonomical level. However, we find that by investigating microbial function - as defined by the genes that are found or associated in the detected microbes - rather than taxonomy, we are able to perform more sensitive analysis and that our results are more easily interpretable. Second, microbiome studies are typically conducted using a single sample per subject. We find that the degree of change in the microbial ecosystem, called volatility, is an important feature of the microbiome and that is linked to severity of stress response. While volatility was coined before in the context of the microbiome, this was only in passing. We were the first to investigate volatility as a feature of the microbiome.

Our research in this thesis reconfirms the existence of the microbiota-gut-brain axis and demonstrates novel metrics that can be used to interrogate the microbiome. We utilize mathematical frameworks originally from geology and classical ecology to bolster our analysis. We show that considering the microbiome as an ecosystem is a powerful model that can help us better formulate our scientific questions and interpret our findings. We argue for strategies to unify bioinformatics methodology in the microbiome-gut-brain axis field in an effort to move towards mechanistic understanding.

Chapter 1 Introduction

Two published manuscripts and one manuscript in preparation were used for this chapter.

Chapter 1.1 largely consists of the following published manuscripts:

Making Sense of ... the Microbiome in Psychiatry

Thomaz F S Bastiaanssen, Caitlin S M Cowan, Marcus J Claesson, Timothy G Dinan, John F Cryan

Published in the International Journal of Neuropsychopharmacology, 2019

DOI: 10.1093/ijnp/pyy067

Gutted! Unraveling the Role of the Microbiome in Major Depressive Disorder

Thomaz F S Bastiaanssen, Sofia Cusotto, Marcus J Claesson, Gerard Clarke, Timothy G Dinan, John F Cryan

Published in Harvard Review of Psychiatry, 2020

DOI: 10.1097/HRP.0000000000000243

Chapter 1.2 largely consists of parts of the following manuscript (in preparation, tentative title)

Dealing with Bugs and Features: A field guide to the statistical analysis of host-microbiome experiments

Thomaz F.S. Bastiaanssen, Thomas P. Quinn, Amy Loughman, John F. Cryan

In preparation, no DOI available.

1.1 Introduction to the Microbiota-Gut-Brain Axis

1.1.1 Introduction

When the Dutchman Antonie van Leeuwenhoek peered through his home-made microscope in the seventeenth century, he dubbed the *kleine diertjens* (tiny animals) he found there *animalcules* (Lane, 2015). The discovery that microorganisms are residing practically everywhere, including in and on humans, had a profound impact on medical knowledge. A short time later, the link between these *small, bloodless animals* and a diarrhea epidemic was suggested by Valk (Valk, 1745). In 1890, Robert Koch published his famous postulates in an attempt to formulate criteria that would establish whether a given microbe causes a given disease (Koch, 1876). Up until recently in medicine, we have regarded microorganisms as undesirable germs to be kept at bay. They were thought to range from pathogenic to harmless to humans and relevant to almost all areas of medicine.

Nonetheless, the disciplines of Microbiology and Psychiatry evolved along distinct trajectories with only a few notable exceptions. Infamously, the psychiatrist Henry Cotton had the teeth of psychiatric patients in his care removed, believing microbes on their teeth to be the source of their illness (Anderson et al., 2017). There is also a report in the British Journal of Psychiatry in 1910 of the successful treatment of melancholia with *Lactic acid bacillus* in 1910 (Phillips, 1910). An early adopter of the idea of microorganisms as beneficial was the 1908 winner of the Nobel Prize in Physiology and Medicine, Metchnikoff. He was convinced of the beneficial effects of fermented milk for “autointoxication” (a rather broad term encompassing a range of negative health outcomes, including fatigue and melancholia; Bested et al., 2013), so much so that it has been reported that he drank fermented milk daily. Despite Metchnikoff’s early hypotheses regarding the potential health benefits of certain bacterial strains, these ideas were largely ignored for the better part of a century.

However, in the last decade, developments in sequencing technology and bioinformatics have allowed in-depth investigations into the composition of complex microbial ecosystems as well as the metabolic and metagenomic potential of such systems. Ventures like MetaHIT (Qin et al., 2010), the Human Microbiome Project

(Methé et al., 2012), the ELDERMET study (Claesson et al., 2012), the Belgian Flemish Gut Flora Project (Falony et al., 2016) and The Dutch LifeLines-DEEP (Tigchelaar et al., 2015) have shed light on the bidirectional relationship between microorganisms and their hosts. This marks a pivotal change in our view of microbes. Not only do we now view microorganisms as a cause of disease, they are also increasingly seen as a cause of health (Bloomfield et al., 2016).

The largest population of microorganisms on the human body resides in the gastrointestinal tract. Known as the gut microbiota, this complex ecosystem is comprised of microorganisms including bacteria, fungi and archaea from over 60 genera (Falony et al., 2016). Recent estimates put the total bacterial count on an average human at around 3.0×10^{13} , which is just more than the estimates of human cells in the body (Sender et al., 2016). In a 70 kg individual, the human gut microbiota would weigh in at an impressive 0.2 kg (Sender et al., 2016). The total genetic material of this mass is known as the microbiome. In terms of genes we are more than 99% microbial, meaning the vast majority of both genes and DNA found in a human originates from microbes (Qin et al., 2010). Perhaps the most surprising development to arise from this field has been the realization that the microbiome plays a key role in the programming of all major body systems, including the brain (Round and Mazmanian, 2009; Diaz Heijtz et al., 2011; Collins et al., 2012; Cryan and Dinan, 2012; Foster et al., 2016; Kundu et al., 2017).

1.1.2 The “Healthy” Microbiome

It is worth reminding ourselves that we are living in a microbial world; microbes were here first and there has never been a time when the brain existed without microbes (Stilling et al., 2014). It makes sense to consider the human host in the context of its environment. While scientific reductionism is a powerful tool, a more holistic systems biology approach has enabled us to more accurately understand complex interactions (Sugihara et al., 2012). In this spirit, the term holobiont, describing the totality of the host and its microorganisms, has gained increasing traction in the field

(Bordenstein and Theis, 2015; Theis et al., 2016). By blurring the borders between otherwise clearly defined organ systems, the holobiont provides a useful concept for understanding the many levels of interaction between the host and its microbiome.

1.1.3 Where it began...

The composition of the microbiome is not only unique to each individual but is also known to differ drastically throughout the host's lifespan. For the most part, colonization of the human gut microbiome is thought to begin at birth, although this notion has become subject to debate based on recent reports of microbial DNA in the placenta and meconium (Stout et al., 2013; Aagaard et al., 2014). While these reports remain controversial (Perez-Muñoz et al., 2017), what is clear is that the neonate is exposed to the vaginal microbiome of the mother during delivery through the birth canal. In contrast, when the newborn is delivered via Caesarean section (C-section), it is exposed to the skin microbiome rather than the vaginal microbiome (Chu et al., 2017). Consequently, the microbiome of children delivered via C-section differs significantly from that of children delivered vaginally (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2016). Other factors, such as prematurity, breastfeeding, the presence of pets, parental smoking, maternal age, weight (especially obesity), and race are also known to impact the developing microbiome (Borre et al., 2014; Bokulich et al., 2016; Levin et al., 2016).

1.1.4 The ageing microbiome

Just as development in early life has been found to parallel gut microbiome development, several age-related diseases have been similarly linked to the state of the microbiome in both animals (Scott et al., 2017) and humans (Claesson et al., 2012). In a study in elderly Koreans, administration of *Lactobacillus helveticus* IDCC3801 improved performance in cognitive fatigue tests (Chung et al., 2014). A decline in microbial diversity is associated with a concomitant increase in microglial activation correlated to brain mass differences in the mouse (Von Bernhardi et al., 2015). This contributes to an age-associated inflammatory response known as “inflammageing”, which in turn has been associated with neurodegenerative

diseases such as Alzheimer's and Parkinson's disease (Franceschi et al., 2007). During ageing, the stability of the microbiome deteriorates (Claesson et al., 2011); however, it is worth noting that we still lack an exact characterisation of the ageing gut microbiome. Decreasing diversity of the gut microbiota has been linked to ageing (Biagi et al., 2010) and age-related impairments like frailty in humans (Claesson et al., 2012; Jackson et al., 2016). In contrast, aged (24-month old) mice exhibit increased microbial diversity compared to younger adult mice (Scott et al., 2017). Intriguingly, the aged gut microbiota composition can also contribute to "inflammageing" (Thevaranjan et al., 2017), the heightened proinflammatory status and decline in adaptive immunity progressively observed in older age (Franceschi et al., 2000). Given the high prevalence of MDD in ageing (Charlton et al., 2018; Beutel et al., 2019), it is tempting to speculate that the microbiome might be at the intersection of ageing and mood, however, this hypothesis needs to be further verified in targeted and large population-based studies (Prenderville et al., 2015). Interventions targeting the microbiome have been found to protect against physiological and neuroimmune changes due to ageing (Boehme et al 2019) and behavioural and cognitive effects of stress (Burokas et al 2017, O'Mahony et al 2019, Provensi et al 2019).

1.1.5 They are what *you* eat

As the infant develops it seems that some of these early factors become less influential. For example, the microbiota of infants born by C-section or natural delivery converges over time, becoming indistinguishable by six weeks of age (Chu et al., 2017; Hill et al., 2017). However, one factor that continues to have a significant impact on microbiota composition throughout the lifespan is the diet of the host (David et al., 2014; Sandhu et al., 2017). In particular, the research shows a stark contrast between the Western diet, with its high sugar, animal fat, and carbohydrate content, in comparison to a Mediterranean diet, which is characterized by increased variety of foods and higher fiber content. The microbiota profile of individuals with these different diets is drastically different (Wu et al., 2011; De Filippis et al., 2016). Although previous studies have segregated different mammalian gut microbiomes

based on their compositions, known as enterotypes, this concept has been challenged and is still in the process of being refined (Costea et al., 2018). While there is still debate over the canonical number of enterotypes in humans, there is a general consensus that a division can be made between an enterotype enriched at the genus level in *Prevotella* and one enriched in *Bacteroides*. Strikingly, this difference can be related to dietary intake. Specifically, fiber-rich diets are associated with the *Prevotella* enterotype, reflecting the role of *Prevotella* species in production of hydrolases specialized for plant fiber degradation (Purushe et al., 2010). *Bacteroides*, on the other hand, are associated with the Western diet (Costea et al., 2018). Specific dietary compounds such as polyphenols, which are known to affect brain chemistry, are also known to influence the microbiome (Filosa et al., 2018; Matarazzo et al., 2018; Donoso et al., 2020).

1.1.6 The microbiota-gut-brain axis

The gut microbiota is known to interact with the brain indirectly, in a bidirectional manner, most likely through a variety of pathways including vagal nerve stimulation, interaction with the immune system and microbial production of human neurotransmitters (see Figure 1; Cryan and Dinan, 2012; Lyte, 2014; Yano et al., 2015; Schirmer et al., 2016; Kennedy et al., 2017). While the precise mechanism of action remains unknown, evidence for bidirectional communication between the microbiome and the brain is clear, and the impact striking.

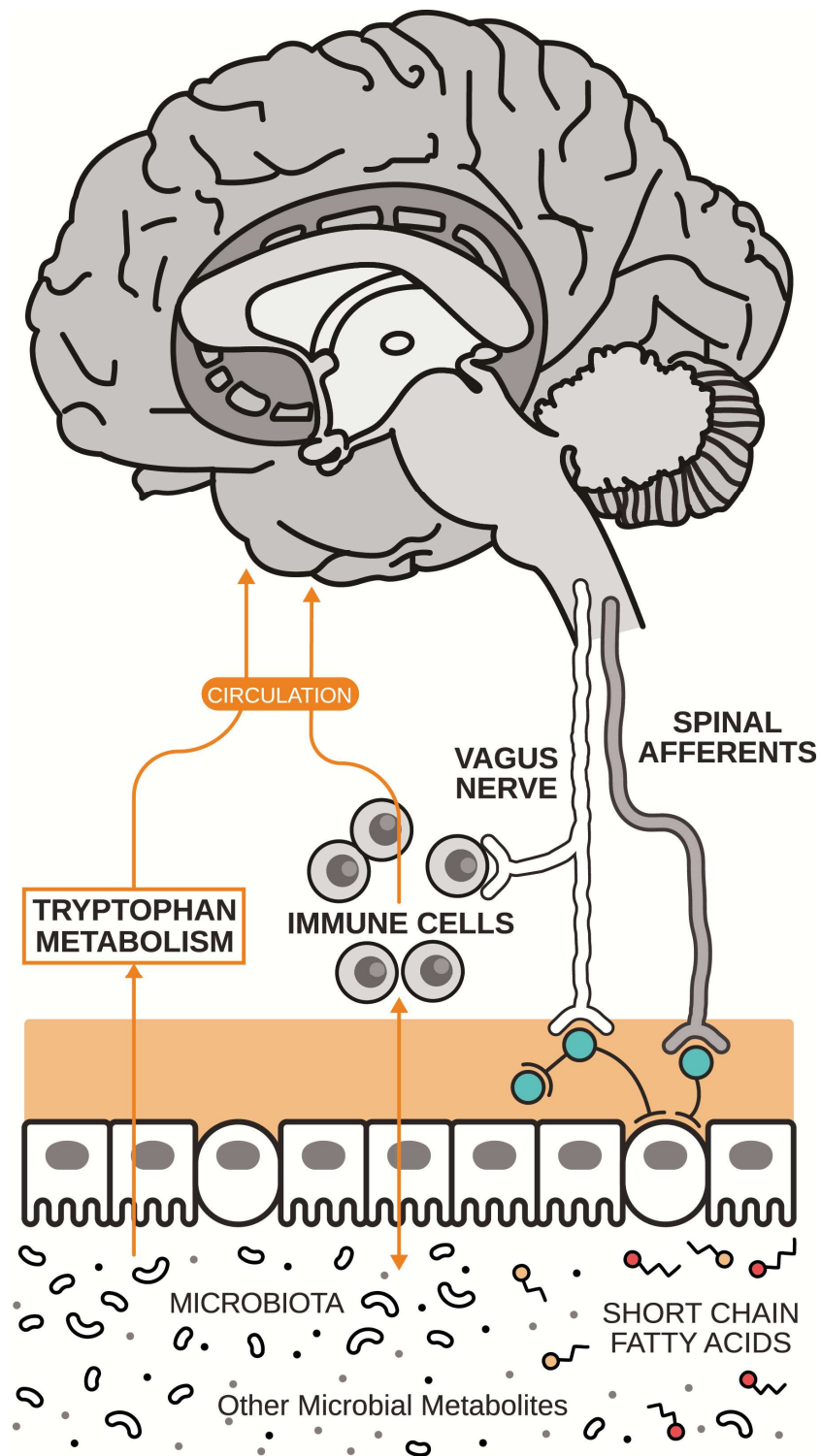


Figure 1.1-1. The microbiota-gut-brain axis Pathways of communication between the gut microbiome and the brain, include vagal nerve stimulation, interaction with short-chain fatty acids, immunoregulatory elements and tryptophan metabolism. In addition, certain microbes are known to produce and secrete human neurotransmitters. Figure adapted from Cowan et al. (2018).

Besides regulating brain function, the microbiome has also been shown to regulate the physical development of the brain (Dinan and Cryan, 2017). For instance, hypermyelination of prefrontal cortex neurons has been observed in the brains of germ-free mice (Hoban et al., 2016). Moreover, the dendrites of neurons in the amygdala and hippocampus of germ-free mice are morphologically distinct to those in control mice (Luczynski et al., 2016). In a recent study, mouse pups born from germ-free mothers were either colonized with microbiota from slow or fast growing human infants (Lu et al., 2018). Pups with microbiota from fast-growing infants showed an accelerated neuronal differentiation when compared to slow-growing humanized and germ-free pups. In addition, slow-growing humanized mice were found to exhibit more signs of neuroinflammation. Finally, the microbiota-derived molecule Pglyrp2, which was determined to cross the blood-brain barrier, has been shown to influence the protein expression profile in the germ-free mouse model (Arentsen et al., 2017).

Completing the circle, not only does targeting the gut microbiome influence the brain, there is research that suggests targeting the brain also influences the gut microbiome. There have been several recent studies indicating that certain pharmaceuticals, especially psychotropic agents, can shape the microbiome (Davey et al., 2012; Davey et al., 2013; Kao et al., 2018; Maier et al., 2018). The best evidence for psychotropic effects on the microbiota have been observed with antipsychotic drugs (Davey et al., 2012; Davey et al., 2013; Kao et al., 2018). In addition, most classes of antidepressants, including the widely-used selective serotonin receptor inhibitors (SSRIs), have also been shown to impact the microbiota, exhibiting antimicrobial activity *in vitro* (Munoz-Bellido et al., 2000; Macedo et al., 2017). These findings are suggestive of a potential whole microbiota-gut-brain axis effect of certain psychotropics, consistent with the effects of stress and psychological state on this axis (Cryan and Dinan, 2012; Moloney et al., 2014; Foster et al., 2017). However, it is difficult to disentangle whether such effects are mediated by changes in signalling from the brain to the gut microbiota or, alternatively, via direct actions of

the drugs on the microbiota. Other tools and models such as brain stimulation and traumatic brain injury are now being used to establish brain to microbiota influences more directly. Brain stimulation research is still very much in the preliminary stages; only one conference abstract has been published, which reported that deep transcranial magnetic stimulation (dTMS) improves symptoms of obesity by modulating gut microbiota (FERRULLI et al., 2018). In a controlled experimental model of stroke in mice, changes in the cecal microbiota were observed within 72 hours after brain damage was induced (Houlden et al., 2016). This work replicates clinical findings from a patient population of Chinese stroke victims who exhibited altered microbiota composition compared to asymptomatic controls (Yin et al., 2015). Together, these studies highlight the substantial influence of the brain over the microbiota, which we are only just beginning to understand.

1.1.7 Tools to Interrogate the Microbiome

Over the years, a plethora of different experimental models have been utilized to investigate the microbiome and its interactions with the host. Here, some of the most common will be discussed. For the most part, mice and rats are used as hosts when modelling the microbiome. While both animals have distinct features when compared to humans, there are many similarities and advantages, making them the preferred models in most studies (Nguyen et al., 2015). However, many other species from drosophila (Leitão-Gonçalves et al., 2017) to zebrafish (Borrelli et al., 2016) and up to primates (Bailey and Coe, 1999; McKenney et al., 2015; Amaral et al., 2017) have also been used to investigate the microbiome. As the field of microbiota-brain interactions matures we can expect that more studies will be carried out in healthy humans and clinical populations, which will further strengthen the conclusions that can be drawn from this line of research.

1.1.7.1 Microbiota depletion: Germ-free animals & antibiotics

As in all aspects of science and engineering, one of the main ways to confirm the importance of a specific process is to remove it and study the consequences. Germ-

free animals represent our best available model for complete removal of all microorganisms. This method has been instrumental in linking the microbiome to many key brain processes and behaviors (Diaz Heijtz et al., 2011; Luczynski et al., 2016; Luczynski et al., 2016). However, given that germ-free animals exhibit such dramatically abnormal neurodevelopment, it is difficult to determine the precise role of the microbiome in said processes (Al-Asmakh and Zadjali, 2015; Luczynski et al., 2016). Moreover, this is an extreme model with limited clinical translation.

While on first glance similar to the germ-free model, antibiotics represent an alternative distinct model to investigate the microbiome (Lundberg et al., 2016). Antibiotics have the advantage that they can be used to knock out/down the microbiota for specified timeframes without affecting neurodevelopmental programming *per se*. However, as antibiotic treatments can negatively impact the animals' health, it is sometimes hard to distinguish the side-effects of the antibiotics from the microbiome-driven effects (Luczynski et al., 2016). Moreover, many antibiotics can cross the blood brain barrier (Nau et al., 2010) and caution is therefore required when interpreting studies of antibiotic-induced microbiota depletion.

1.1.7.2 “Friends with benefits”: Prebiotics, probiotics, synbiotics & psychobiotics

While disruption of the microbiome can have a negative effect on the host, supplementing the microbiome has been used as a strategy to optimize host performance. Introducing probiotic microbes that are known or suspected to be beneficial is an intuitive way to investigate the relationship between the host and the microbiome. Here, it is important to note that it is likely not just specific microbes that may be beneficial, but the collateral effects of that strain on the microbial ecosystem in given niches (Duran-Pinedo and Frias-Lopez, 2015). Although the term probiotic has gained substantial public attention and become part of the wider vocabulary, it is important to clarify that many commercially available strains marketed as probiotics have never been tested in clinical trials and therefore by definition would not meet the criteria of conferring a health benefit.

Prebiotics represent a more general way to alter microbiome composition, essentially providing nutrients to encourage the growth of beneficial microorganisms (Gibson et al., 2017). However, prebiotics are considered less specific than probiotics as there is little control over which microorganisms will metabolize the prebiotics and which will proliferate. A growing body of work is now focused on combining prebiotics and probiotics to develop synbiotics (Ford et al., 2014). Finally, and most recently, the term psychobiotics has been introduced to describe targeted microbiome interventions with a beneficial effect on *mental health*, which are of particular interest to the study of psychiatric disorders (Dinan et al., 2013; Sarkar et al., 2016; Anderson et al., 2017). Overall, these approaches are appealing because they can be introduced in food and drink and therefore provide a relatively non-invasive method of manipulating the microbiota. While these studies show the potential of probiotics, negative studies have demonstrated that similar probiotic treatments can vary in effectiveness, suggesting that there are more factors at play than just the specific probiotic strain used (Hojsak et al., 2015 ; Mazurak et al., 2015). This conforms with the understanding that the behavior of a microbial strain is dependent on its metabolic, microbial and host environment (Succurro et al., 2018).

1.1.7.3 Fecal microbiota transplantation (FMT)

The concept of fecal microbiome transplantation (FMT) as a therapeutic intervention is disrupting Western medicine completely. The procedure involves introducing fecal microbiota from a selected donor to the gastrointestinal tract of the recipient, with the aim of making the recipient microbiome more similar to the donor (Borody and Khoruts, 2012). When used as a therapeutic intervention, donors must be screened to ensure they are healthy, as phenotypes like obesity and depression have been shown to be transferable via FMT, at least in rodents (Turnbaugh et al., 2006; Kelly et al., 2016). FMT used in a preclinical setting can involve deliberately unhealthy donor phenotypes. The realization that patients with recurrent *Clostridium difficile* infection have a good chance to recover after FMT treatment represents an arguably

non-invasive and cheap approach to an otherwise difficult to treat disease (Gianotti and Moss, 2017). Moreover, the potential of FMT as a clinical and experimental tool is reflected in the application of this approach to treat a wide variety of diseases (e.g. irritable bowel syndrome, steatohepatitis, ulcerative colitis, and even autism Pinn et al., 2015; Ren et al., 2015; Kang et al., 2017; Zhou et al., 2017) and investigations of the effects of inter-species FMT from specific clinical populations to experimental rodents (Arrieta et al., 2016). Intriguingly, FMT from young donors to middle-aged recipients has even been used to extend the lifespan of killifish (Smith et al., 2017).

1.1.7.4 Cross-sectional studies

One of the most widely used methods to study the microbiome in humans is to assess microbiome composition across cohorts of clinical patients and matched controls. Thanks to the increasing number of such studies including the microbiome in their measurements, there are a large number of databases available for interrogation, such as the Human Pan Microbial Communities Database (HPMCD; Forster et al., 2016) and the NIH Human Microbiome Project (HMP; The N. I. H. H. M. P. Working Group et al., 2009). Here, it is important to note that it is often problematic to pool measurements from different databases together because the exact techniques used for extraction and processing of microbial genetic material account for a large part of the variation between samples (Clooney et al., 2016).

1.1.8 Linking the Microbiome to Psychiatric Disorders

Given the many modes of communication between the brain and the gut microbiome, it is not difficult to imagine the impact the gut microbiome has on host mental health and illness. Here, we first discuss the role of the gut microbiome in stress regulation, as stress is one of the most potent risk factors for psychiatric illness. We then briefly discuss the current state of the evidence linking the microbiome to various psychiatric disorders, from developmental disorders to mood, anxiety, and eating disorders.

1.1.8.1 The microbiome and stress

There is a robust association between stress, which is associated with activation of the hypothalamus-pituitary-adrenal (HPA) axis, and the state of the microbiome (for reviews, see Moloney et al., 2014; Gur et al., 2015; de Weerth, 2017; Foster et al., 2017; Bastiaanssen et al., 2018; Cryan et al., 2019). A number of studies have demonstrated that stress alters the composition of the microbiota in a range of different hosts, from rats and mice (Gareau et al., 2007; O'Mahony et al., 2011; Golubeva et al., 2015; Bharwani et al., 2016; Burokas et al., 2017) to Syrian hamsters (Partrick et al., 2018), pigs (Mudd et al., 2017) and non-human primates (Bailey and Coe, 1999; Bailey et al., 2011).

In the other direction, the gut microbiome also regulates the stress response. In a seminal study, Sudo et al. (2004) elegantly demonstrated that germ-free mice exhibit elevated HPA axis responses to stress as measured by adrenocorticotrophic hormone and corticosterone. The HPA axis response was found to be normalized by colonization with a probiotic species but exaggerated by colonization with an enteropathogen in the same study. Similarly, probiotics have been shown to reverse stress effects in many studies using various animal models (Gareau et al., 2007; Desbonnet et al., 2010; Bravo et al., 2011; Ait-Belgnaoui et al., 2012; Barouei et al., 2012; Liang et al., 2015; Cowan et al., 2016; Bharwani et al., 2017; Callaghan, 2017). Promisingly, there is analogous evidence that probiotics promote stress resilience or reduce stress-induced physical symptoms and cognitive deficits in humans (Diop et al., 2008; Langkamp-Henken et al., 2015; Kato-Kataoka et al., 2016; Allen et al., 2017; Wang, 2017; Papalini et al., 2018). Finally, certain prebiotics have also been shown to protect against stress-induced effects on the microbiome, physiology and behavior (Tarr et al., 2015; Burokas et al., 2017; Provensi et al., 2019).

1.1.8.2 Major depression

There is strong (and continually mounting) evidence that the microbiome plays a role in major depression (Foster and McVey Neufeld, 2013; Dash et al., 2015). Germ-free mice display reduced depressive-like behavior; in the forced swim test of behavioral despair, germ-free mice will continue swimming or attempting to escape

an inescapable pool for longer than control mice (Zheng et al., 2016), while both probiotic and prebiotic treatments have been shown to reduce depressive-like behavior in rodent models (Desbonnet et al., 2010; Bravo et al., 2011; Burokas et al., 2017). These studies seem to hold translational value, with several systematic reviews indicating that probiotics effectively improve mood in humans (Huang et al., 2016; Pirbaglou et al., 2016; Wallace and Milev, 2017). It is worth noting though that one such systematic review found that benefits were limited to those with mild to moderate depression (i.e. healthy individuals did not significantly benefit; Ng et al., 2018) which, alongside probiotic strain differences, may explain some of the conflicting findings in the attempts to translate probiotic effects to humans (Allen et al., 2016; Kelly et al., 2017).

Clinically, several studies have found an altered microbial composition in patients with major depression (Naseribafrouei et al., 2014; Jiang et al., 2015; Kelly et al., 2016; Zheng et al., 2016). Of note, two studies reported a reduction in the relative abundance of *Faecalibacterium* (Jiang et al., 2015; Zheng et al., 2016), mirroring the results described earlier for bipolar disorder (Evans et al., 2017). Jiang et al. (2015) went further to identify a negative correlation between the severity of depression and the prevalence of *Faecalibacterium*. Another study reported lower levels of *Bifidobacterium* and *Lactobacillus* in depressed patients (Aizawa et al., 2016). Strikingly, when the gut microbiome of depressed humans has been transferred to either rats or mice via FMT the recipient animals exhibit greater depressive- and anxiety-like behavior compared to those that received FMT from healthy humans (Kelly et al., 2016; Zheng et al., 2016).

1.1.8.3 Anxiety disorders

There is clear preclinical evidence to support a link between anxiety and the microbiome (Foster and McVey Neufeld, 2013; Malan-Muller et al., 2018). Germ-free mice and zebrafish exhibit reduced anxiety-like behavior (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Clarke et al., 2013; Davis et al., 2016), although germ-free rats

exhibit more anxiety-like behavior compared to conventionally colonized controls (Crumeyrolle-Arias et al., 2014). Anxiety-associated microbiome differences have also been observed between strains of mice, with the anxious BALB/c having a distinct microbiome profile compared to the more resilient Swiss Webster strain (Bercik et al., 2011). Furthermore, FMT from one mouse strain to the other was sufficient to partially transfer the respective behavioral phenotypes (i.e., BALB/c mice given NIH Swiss microbiota became less anxious, whereas NIH Swiss mice given BALB/c microbiota became more so).

Additional preclinical studies have shown that probiotic and prebiotic treatments can reduce anxiety-like behaviours in rodents (e.g. Bravo et al., 2011; Burokas et al., 2017). Unfortunately, very few studies have examined the relationship between anxiety and the microbiome in clinical populations. A single, small study of a South African population revealed specific phylum-level differences in the microbiome for those diagnosed with post-traumatic stress disorder (PTSD) in comparison to trauma-exposed controls (Hemmings et al., 2017). Aside from this correlational study, there have been two small intervention studies showing that probiotics reduce self-reported anxiety in healthy individuals (Messaoudi et al., 2011) and in a clinical group presenting with chronic fatigue syndrome (Rao et al., 2009).

1.1.8.4 Obsessive-compulsive disorders (OCD)

While there have been no direct investigations (as of yet) into the microbiome in obsessive-compulsive disorder (OCD) patients, several researchers have speculated that there may be a link (Rees, 2014; Turna et al., 2016). This hypothesis is based on two lines of observation. First, it has been noted that many of the risk factors for onset of OCD are also known to disrupt the microbiome, including stress, pregnancy and antibiotic use (Rees, 2014). Second, there is preclinical evidence that OCD-like behavior in rodents (frequently measured using the marble burying test, which aims to assess repetitive, compulsive behaviors, one of the core symptoms of OCD) can be modified by microbial treatments, including germ-free environments and probiotic treatments (Nishino et al., 2013; Kantak et al., 2014; Savignac et al., 2014). Notably,

the deer mouse, or *Peromyscus maniculatus*, is used as an animal model for OCD. The deer mouse is a nest builder. Naturally, a large proportion of deer mice will display a behaviour where they build abnormally large nests (Wolmarans et al., 2016). Indeed, large nest building deer mice have been shown to respond to selective serotonin reuptake inhibitors (SSRIs), which are commonly prescribed for OCD. Recently, the large nestbuilding phenotype was shown to have a differing microbiome composition compared to its normal nestbuilding counterpart (Scheepers et al., 2020).

1.1.8.5 Eating disorders

The gut microbiome has also been linked to diet-induced obesity (Torres-Fuentes et al.). Obese individuals exhibit differences in microbiota composition (Ley et al., 2005; Turnbaugh et al., 2006; but see also Sze and Schloss, 2016). Importantly, a causal contribution of the microbiome to diet-induced weight gain has been demonstrated using mice with a humanized microbiome (Turnbaugh et al., 2009). In these mice, switching from a plant-based diet to a Western-style diet caused rapid shifts in the microbiome composition (within 24 hours) and subsequent weight gain. Furthermore, the increased adiposity associated with the Western diet could then be transferred to naïve mice via FMT. Offering hope that we might utilize the microbiome to enact positive weight changes as well, it has been hypothesized that the microbiome may contribute to weight loss following bariatric surgery, based on evidence that such surgeries induce microbiome alterations in both humans and rodents (Peat et al., 2015; Torres-Fuentes et al., 2017).

In patients suffering from disorders that are associated with altered eating habits, it will continue to be difficult to disentangle the direction of microbiome-mental health relationships. It is intriguing to consider this problem; is eating behavior “manipulated” by an altered microbiome (as has been suggested by some, e.g. Alcock et al., 2014), does eating behavior drive microbiome changes and thereby alter gut-brain communication, or both? When considering this question, it is worth noting

that changes in eating habits are not limited to eating disorders but are observed across a variety of psychiatric disorders (including anxiety, ADHD, ASD, depression; Yannakoulia et al., 2008; Ptacek et al., 2014), while epidemiological studies show that healthy dietary patterns are associated with better mental health (O'Neil et al., 2014). It is therefore an important question that deserves ongoing attention. Regardless of the initial cause of these disruptions, the opportunity to utilize dietary or other microbiome-targeting interventions to improve mental health holds great appeal and scientific potential.

1.1.9 Linking the Microbiome to Neurological Disorders

1.1.9.1 Alzheimer's disease (AD)

In addition to being involved in the general phenomenon of inflammaging, the gut microbiome has also been found to be involved in specific age-related illnesses. In patients with Alzheimer's disease (AD), Hill et al. (2014) reported a correlation between colonization of certain pathogenic microbes such as *Toxoplasma* and *Clamydophila pneumoniae* and progression of the disease. Furthermore, patients suffering from AD were shown to have a less diverse microbiome with distinct compositional differences when compared to the healthy microbiome (Vogt et al., 2017). In the same study, the researchers theorize about the high prevalence of pro-inflammatory lipopolysaccharide producing gram-negative bacteria such as *Bacteroides* in AD patients and their role in pathogenesis (Cattaneo et al., 2017). In a large Chinese cohort, patients with mild cognitive decline had a distinct microbiome from patients suffering from AD (Liu et al., 2019). Promisingly, a modified ketogenic diet targeting the microbiome has been shown to ameliorate some of the mild cognitive decline effect of AD (Nagpal et al., 2019). Finally, germ free or antibiotic-treated transgenic AD mouse models fail to develop plaques (Harach et al., 2017; Minter et al., 2017).

1.1.9.2 Parkinson's disease (PD)

There is a growing emphasis on the role of the gut-brain axis in the onset of Parkinson's disease (PD; Dinan and Cryan, 2017; Perez-Pardo et al., 2017; Elfil et al., 2020). A number of studies have shown alterations in the microbiome in PD (Scheperjans et al., 2014; Keshavarzian et al., 2015; Heintz-Buschart et al., 2018; Qian et al., 2018; Sun et al., 2018). When mice were colonized with the microbiota of PD patients via FMT, they developed motor deficits and neuroinflammation, two hallmark symptoms of PD (Sampson et al., 2016). Additionally, symptoms improved when the mice were treated with antibiotics. In rats, overexpression of alpha-synuclein cooccurred with alterations in the gut microbiome (O'Donovan et al., 2020). Large-scale investigations using the extensive patient records in Denmark and Sweden have shown that vagotomy (or more specifically truncal vagotomy), which removes one of the major routes for microbiota to brain communication, is protective against PD (Svensson et al., 2015; Liu et al., 2017).

1.2 Introduction to Microbiome Bioinformatics Analysis

1.2.1 Introduction

With the advent of high-throughput sequencing, the gut microbiome has become a popular subject of investigation. As part of these investigative efforts, it has become increasingly clear that the microbiome is in constant bidirectional communication with the host, and that both systems influence each other on multiple levels. For instance, the human gut microbiome has been shown to differ between individuals on the basis of dietary factors, physical health, age, medications, and even psychological health (Consortium, 2012; Cryan et al., 2019; Vujkovic-Cvijin et al., 2020). Analysing and interpreting microbiome experiments can be challenging for various reasons. Some factors include the vast range of scientific fields relevant to the microbiome--each with their own unique research culture and norms--and the sheer number of specialist software tools needed to pre-process and analyse the associated data. In this section, we present an overview of the various methods used to analyse, interpret, and visualise microbiome studies. Figure 1.2-1 shows a representation of what a typical microbiome analysis workflow may look like.

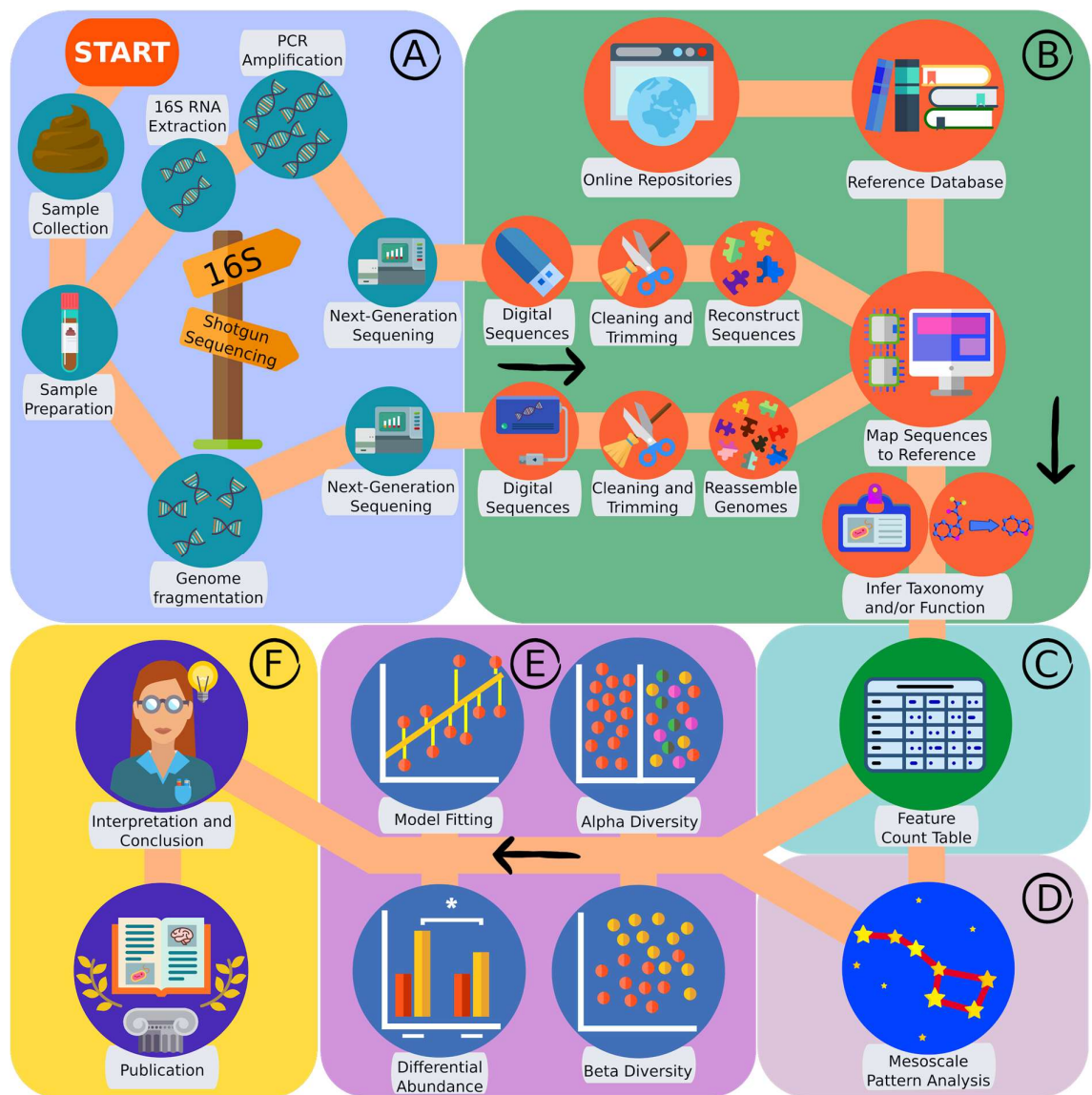


Figure 1.2-1. From Stool to Story. Overview of what a typical gut microbiome analysis may look like. A) Shows the pre-digital part of the pipeline. Genetic material is isolated and digitized, either using the 16S or shotgun sequencing approach. In B) the digitized reads are annotated and based on taxonomy and/or function. In C), the features are tallied up into count tables. In D), mesoscale patterns, or patterns within the data that are larger than features but smaller than the whole such as functional modules, are identified. In E) features of the microbiome are assessed statistically. Finally, in F), the features are interpreted and presented for peer-review.

1.2.2 16S amplicon vs. shotgun sequencing

Generally speaking, two methods of microbiome sequencing are widely used: (1) 16S or amplicon sequencing, which includes methods where an evolutionarily preserved genomic sequence is targeted and sequenced, and (2) whole genome shotgun sequencing, where all genetic material in a sample is targeted and sequenced.

1.2.2.1 Pre-processing 16S sequencing data

The analysis of 16S sequencing typically begins by trimming reads, filtering them for quality based on a threshold, and removing chimera sequences. Then, a table of either operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) is generated. The philosophy and process behind these two units differ meaningfully, and this has been comprehensively written about elsewhere (Callahan et al., 2016). For all but the most theoretical purposes, both OTUs and ASVs can be seen as the highest taxonomic resolution that a specific method can distinguish. Roughly speaking, they can be viewed as analogous to species or genera. Although OTUs and ASVs are technically distinct, the two are interchangeable concepts when it comes to downstream statistical analysis.

After generating a table of OTUs or ASVs, the next step is to assign taxonomy. In most cases, this is done by use of a reference database. Several such databases exist, and some are better curated than others. At time of writing, the SILVA database is widely regarded as the most accurate and extensive (Quast et al., 2012). Although the Greengenes database is still often used, it has not been updated since 2013.

1.2.2.2 Pre-processing shotgun sequencing data

In the case of shotgun sequencing, it is also common to filter and trim reads in a fashion analogous to 16S data. Apart from this, non-microbial genetic material needs to be filtered out. This is often done by removing all reads that map to a reference genome of the host organism, as well as any other genomes that may be contaminants (e.g., plant genetic material from diet).

1.2.2.3 The count table

Although 16S/amplicon and shotgun sequencing differ widely in execution, the type of data that is obtained tends to converge downstream in the analysis. After pre-processing, both 16S and shotgun sequencing methodologies yield a *count table*. A count table shows how many observations (i.e., counts) there were for each feature (e.g., microbe, function, gene, etc.) per sample. By convention, a count table will have features as columns and samples as rows. Although many software tools assume this organisation, there are notable exceptions, so it is always worth checking the software before proceeding with an analysis. It is tempting to directly correspond a count to a biological instance of a feature in a sample, but due to biases inherent to metagenomic sequencing (McLaren et al., 2019), raw counts should not be used with initial pre-processing (e.g., normalization). The remainder of this guide assumes the use of a count table, though some of the methods presented -- notably, log-ratio transformation-based methods -- will perform identically for counts and proportions.

1.2.2.4 Rare features and Rarefaction

Before the microbiome analysis starts, it is common to filter out rare features. Commonly, features that are only detected in a certain percentage of samples are removed. This is referred to as *prevalence filtering*. Similarly, features that are only detected in low levels can be dropped here. This is referred to as *abundance filtering*. In rare cases, features can be filtered out based on other metrics, such as variance (Guyon and Elisseeff, 2003). Importantly, features should not be filtered based on their association with a phenotype, as this could bias the p-value estimates of downstream statistical tests.

The total number of observations recorded for each sample in a count table depends on the sequencing depth of the assay. Rarefaction is the practice of randomly removing observations from a sample until all samples have the same number of observations. However, it has been described as an unnecessary and potentially counterproductive measure (McMurdie and Holmes, 2014). It is more conventional now to address inter-sample differences in sequencing depth through effective

library size normalization or log-ratio transformation (Gloor et al., 2017). One notable exception is *alpha diversity analysis*, as discussed below.

1.2.3 Linking the microbiome to host features

1.2.3.1 Diversity indices

The microbiome is a complex ecosystem. The analysis and visualisation of the microbiome can be qualitatively distinct from other high-throughput sequencing data. Although the data arise from a molecular biology assay, several of the statistical approaches used in microbiome analysis originate from other fields, such as ecology. This makes microbiome science a clear beneficiary of interdisciplinary research.

Diversity, as popularized in ecology, is a way to quantify and understand variation in microbiome samples. Classically, diversity is separated into three related types: Alpha, Beta, and Gamma diversity (Hsieh et al., 2016). Alpha diversity refers to the degree of variation within a sample. Beta diversity refers to the degree of variation between samples. Gamma diversity refers to the total diversity in all samples (which can be thought of as the Alpha diversity of all samples combined). In practice, Gamma diversity is rarely used.

1.2.3.2 Alpha diversity – the diversity within samples

There are many measures of alpha diversity, which can make alpha diversity confusing to understand. It is helpful to classify alpha diversity measures along two axes: the *Hill number* (0, 1, or 2) and whether it is *phylogenetic* (yes or no).

Regarding the first axis, alpha diversity measures can be understood as being the result of a unifying equation in which a single parameter--called the Hill number--acts to vary the meaning for the equation, and thus define the alpha diversity measures. Every number gives a different alpha diversity metric. In practice, three Hill numbers are most often used: 0, 1 and 2. The number 0 defines *Richness*, or how many different features a sample has. The number 1 defines *Evenness*, or how equally the features in a sample are represented (equivalent to Shannon entropy). The number

2 defines Simpson's Index, or the probability that two features picked at random do not have the same name (as a probability it is bounded by 0 and 1).

Regarding the second axis, other *phylogenetic diversity (PD) measures*, like Faith's PD, extend alpha diversity by taking into account the coverage of all features (e.g., bacteria) on a phylogenetic tree. Typically, the more of the tree that is represented in a sample, the higher the diversity. Figure 1.2-2 illustrates a classification of several popular alpha diversity measures.

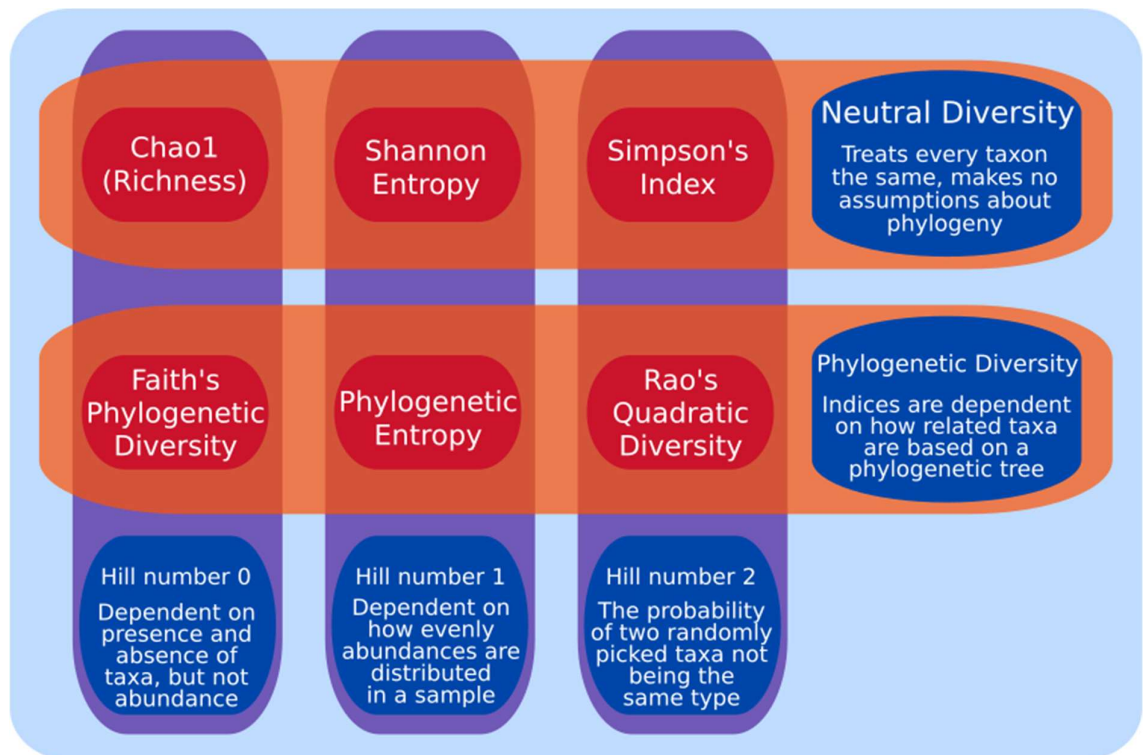


Figure 1.2-2. Alpha Diversity metrics are related to each other. Commonly used alpha diversity metrics in the microbiome field can be mapped on two axes. Here, we show the hill number used on the x-axis and whether the index considers phylogeny on the y-axis.

1.2.3.3 Statistical considerations with Alpha diversity

Alpha diversity is used to summarize the entire microbiome composition as a single number (Hsieh et al., 2016; Hsieh and Chao, 2017). It is common to model alpha

diversity as a dependent variable, using sample meta-data as the predictors. When this is done, the literature has shown that a lower Alpha diversity is often associated with worse host health outcomes (Ma, 2020). However, it should by no means be taken as principle that a higher Alpha diversity is strictly “better”, as there are many examples where elevated Alpha diversity indicates an abnormal or even unhealthy host state. For instance, in infants there is a high selection pressure on certain microbes, such as numerous species in the genera *Bifidobacterium* and *Lactobacillus* (Yang et al., 2019). Here, an increased Alpha diversity could indicate a lowered selection pressure, which could be indicative of health issues (Hill et al., 2017).

There are at least 3 issues to consider when using alpha diversity for microbiome data analysis. First, all alpha diversity measures are sensitive to transcript-level measurement biases such as PCR bias (McLaren et al., 2019). This is recognized as a critical limitation of alpha diversity that cannot be resolved unless the PCR bias factors are already known *a priori* (McLaren et al., 2019). Second, some alpha diversity measures will change depending on the total number of observations (i.e., counts) recorded for a sample. It is often appropriate to “normalize” away differences in sequencing depth before comparing alpha diversity between samples. This can be done by dividing out total counts to get proportions (e.g., in the case of Shannon entropy), or by performing rarefaction (no longer recommended, as discussed above). Both procedures will equalise the number of observations between samples, so that they can be compared fairly. In fact, many alpha diversity software tools will perform this “normalization” step automatically. Third, all alpha diversity measures are sensitive to the number of rare taxa that get observed in samples, and thus are sensitive to sequencing depth. Failure to record the presence of a rare taxa, when it is in fact present, can make a sample appear less diverse than it is.

It is important to keep in mind these 3 issues when interpreting the results from an alpha diversity analysis. For example, in the case of very low microbial load due to, say, an antibiotics course, alpha diversity may appear higher than expected (Elokil et al., 2020). This seemingly paradoxical phenomenon can be better understood when

considering that there is a limited amount of sequencing material during the sequencing process, regardless of method used. In the case of an abundance of microbes, the most prevalent ones will *use up* most of the sequencing reagents, leaving little for the rarer taxa to be sequenced. In the case of a low bacterial load, there are no prevalent microbes to take up most of the material and thus the rarer taxa that happen to be in the sample will have a much higher likelihood to be sequenced, thus inflating the calculated diversity.

1.2.3.4 Beta diversity – the diversity between samples

Beta diversity refers to the degree of difference between two microbiomes (Goodrich et al., 2014; Bastiaanssen et al., 2021). It is worth appreciating the assumptions and limitations that come with describing the total difference between two complex ecosystems as a single number. There are many ways to measure the “difference” between two samples, and each one imparts a unique perspective on the data. In principle, one could use any dissimilarity or distance measure. Three common difference measures are:

1.2.3.4.1 Jaccard’s Index

This is a similarity measure that simply describes the proportion of all features that are shared between two samples, without taking abundance into account. As such, one could interpret Jaccard’s Index as the fraction of *unique taxa (not abundances)* shared by two samples. If two samples have exactly the same microbe taxa, the Jaccard index will be 1. In the case that two samples share no microbe taxa, the Jaccard index will be 0. Subtracting Jaccard’s index from 1 makes it the Jaccard Distance measure.

1.2.3.4.2 Euclidean Distance

This is the geometric distance derived by applying the Pythagorean theorem, using every microbe as a separate dimension. It is computed by taking (the square root of) the sum of the squared differences in bacteria abundance. As in geometry, the minimum Euclidean distance is 0 while the maximum is unbounded. Euclidean Distance satisfies the triangle inequality, making it useful for certain geometric analyses, such as *volatility analysis* as discussed below. A related measure called

Aitchison Distance is the Euclidean distance between log-ratio transformed data. This distance has a favorable property known as sub-compositional dominance (i.e., the removal of a taxa feature will never make two samples appear further apart), and is also equivalent to taking the Euclidean distance between all pairwise log-ratios (Aitchison et al., 2000).

1.2.3.4.3 Bray-Curtis Dissimilarity

This dissimilarity measure is similar to Jaccard's Index in that it ranges from 0-to-1, while also being similar to Euclidean distance in that it is computed from the differences between abundances. Bray-Curtis is calculated by summing the difference in abundance between each bacteria taxa, and dividing it by the total microbial abundance of the two samples. Thus, one could interpret Bray-Curtis as the fraction of *abundances* (not *unique taxa*) unshared by two samples.

The three common difference measures listed above make use of bacteria presence or abundance without considering the phylogenetic relationship between the bacteria. Just as we can make alpha diversity *phylogenetic*, we can do the same with beta diversity.

1.2.3.4.4 UniFrac

This distance makes use of phylogenetic information to measure the difference between samples. There are (at least) two types. The Unweighted UniFrac Distance considers the *branch lengths of the phylogenetic tree* along with bacteria *presence*, and is defined as the sum of branch lengths unshared between the samples divided by the sum of branch lengths present in either sample. This measure has some analogy to Jaccard Distance in that an unweighted UniFrac distance of 1 means the two samples share no bacteria taxa in common. The Weighted UniFrac Distance further considers bacteria *abundance*, and weighs each branch length in the Unweighted UniFrac formula by per-sample proportional abundances.

1.2.3.4.5 PhILR

This method uses a log-ratio transformation called the isometric log-ratio (ILR) transformation which uses a phylogenetic tree to recast the microbiome variables as a series of log-contrasts called "balances" (Silverman et al., 2017). PhILR offers 2

weighting options called taxon weighting and branch weighting. When both are disabled, the PhILR beta diversity is equivalent to Aitchison distance, although its use of phylogeny-based coordinates may yield a more interpretable ordination of the data (for example, via a principal coordinates plot). The taxon weighting provides a compositionally robust alternative to weighted Jaccard or Bray-Curtis measures, while the branch weighting provides a compositionally robust alternative to UniFrac measures.

Figure 1.2-3 illustrates a decision tree that we the authors use when selecting a beta diversity measure. As with alpha diversity, it is sometimes helpful to compare and contrast the results from multiple measures of beta diversity.

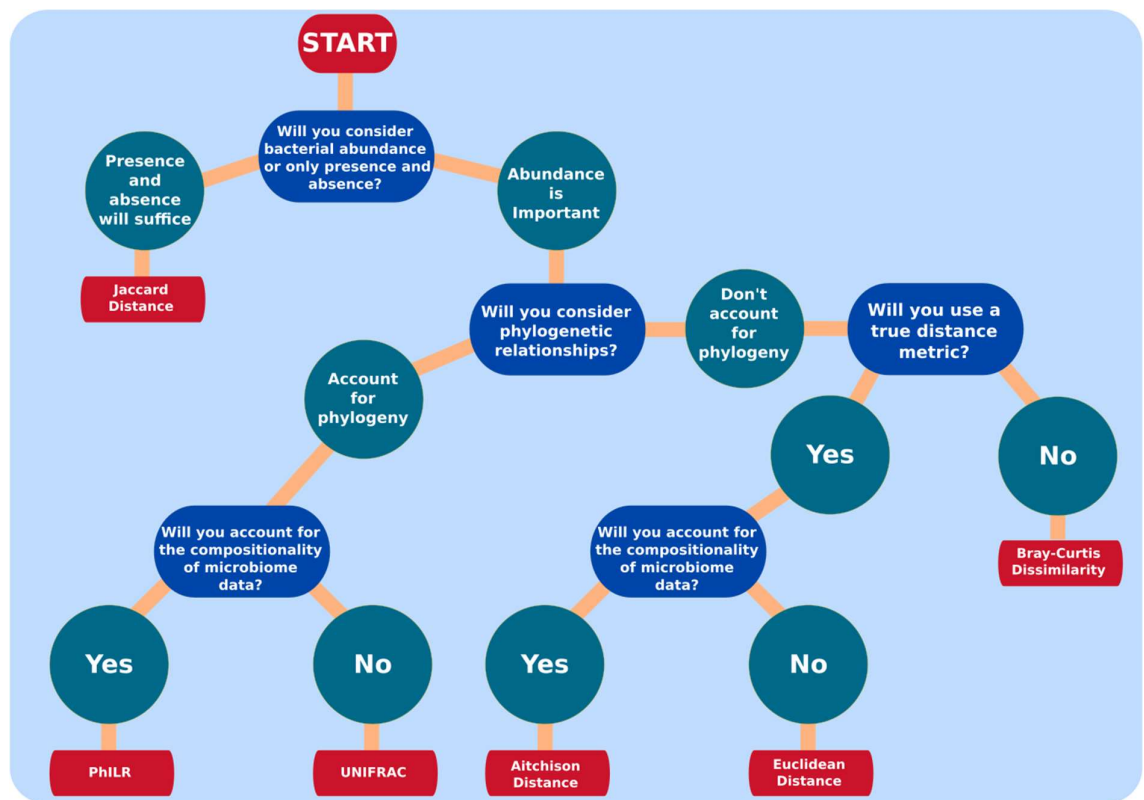


Figure 1.2-3. Decision tree featuring common Beta diversity indices. Different Beta diversity indices are more suitable depending on the needs of the researcher. This decision tree recommends an index based on three common criteria: The need to consider abundance, phylogeny and whether it needs to be a true distance metric (i.e. whether the metric needs to be compatible with geometry).

1.2.3.5 Statistical considerations with Beta diversity

There are two general strategies used to assess beta diversity:

- (1) Qualitative – visualization of samples plotted across an ordination of the data, such as a principal components analysis (PCA) or principal coordinates analysis (PCoA)
- (2) Quantitative – explicit modelling of PCA/PCoA axes as a dependent variable, using the sample meta-data as the predictors, or some other formal comparison between the group centroids

When discussing Beta diversity, it is important to consider that microbiome data are compositional. This is because some common difference measures can have an irregular behavior when applied to compositional data (most notably, Euclidean distances). Fortunately, the study of compositional data has allowed for the development of tools and transformations that enable us to work with compositional data in virtually the same manner as regular data. In the case of Beta diversity, the alternative to Euclidean distance is Aitchison distance (Aitchison et al., 2000). One clear advantage of Aitchison distance, which applies to the unweighted PhILR distance too, is that -- unlike alpha diversity and other beta diversities -- it is unaffected by PCR bias (McLaren et al., 2019).

We note here that there is an interesting parallel between compositional data and the probability vectors routinely studied in information theory. Similar to how Shannon entropy can be used to measure alpha diversity, other informatic metrics like Kullback-Leibler divergence could feasibly be used to measure beta diversity (Erb and Ay, 2020). Although these metrics are not commonplace in microbiome analysis, they are often used in machine learning, and may be more robust for the analysis of *amalgamated data*, for example genus-level or family-level counts (Quinn and Erb, 2020).

1.2.3.6 Volatility

The microbiome is a dynamic ecosystem and undergoes constant change. The degree of change in the microbiome over time is called *volatility*, which is inversely related to *stability*. It can be helpful to think of volatility as a change in sample diversity (alpha

or beta) over time. In a neutral setting, without intervention, a higher volatility is generally considered to be associated with negative health outcomes (Bastiaanssen et al., 2021). The term was first coined in the context of the microbiome in a study noting that the microbiome of patients with inflammatory bowel disease tend to change more over time than those of healthy controls (Halfvarson et al., 2017). One way to calculate volatility is to measure the beta diversity between two or more time points corresponding to the same host. When measuring volatility in this fashion, it is especially useful to choose a beta diversity metric that is also a distance (i.e., follows triangle inequality, like PhiLR or Aitchison distance).

1.2.3.7 Differential feature abundance

1.2.3.7.1 Taxa and Genes

Differential abundance (DA) analysis is perhaps one of the most popular microbiome analyses. Like alpha and beta diversities, there are many approaches to measuring DA. Most methods follow the same general pattern: (a) applying a normalization to correct for variation in sequencing depth; (b) performing a univariate statistical test for each taxa as a dependent variable with the sample meta-data as predictors; and (c) adjusting the p-values for multiple testing, for example using Bonferroni, Storey's q-value or Benjamini-Hochberg.

1.2.3.7.2 Functions

Another line of investigation that starts at this point is functional inference. There are two general strategies to measuring whether the functions of the measured taxa (or genes) associate with the sample meta-data. Both approaches require some external database that assigns functions to the taxa (or genes), which we will term a *functional database*.

(1) Primary analysis: In this approach, the functional database is used to score each function based on the taxa or gene abundances. This produces a functional count table, which is simply a count table tallying the occurrence of functions rather than taxa or any other feature. Then, one could proceed with a routine statistical analysis

that uses the functional scores as a dependent variable, as one would for a microbial count table.

(2) Secondary analysis: In this approach, a DA analysis is first performed on the taxa or genes, and then the functional database is used to summarize the DA results. In the simplest case, the DA results can be dichotomized into significant or non-significant, and functional status can be dichotomized as present or absent. For each function, one could perform a Fisher exact test (or similar) to measure whether that function is over-enriched among the significant taxa or genes (Irizarry et al., 2009). Gene set enrichment analysis (GSEA) is a popular generalization of this concept, and is commonplace in gene expression analysis (Irizarry et al., 2009; Chong et al., 2018).

The way in which one gets to the functional count table depends on the type of sequencing. In the case of 16S, Piphillin and PICRUSt2 are available. Both of these tools infer what the metagenome of a sample might look like by comparing all input 16S sequences to a functional database (currently KEGG and MetaCyc) of fully sequenced microbial genomes and building a functional count table based on the functions present in these reference genomes. While these methods remain inferential, they do provide a reasonably accurate view of the functional potential of a microbiome. In the case of shotgun sequencing we still infer function, but since we do so from evidence from the genetic sequences in our sample, we only need to identify genes and annotate them, often by using a database. Tools like HUMAnN2 in the biobakery suite are typically used to generate a functional count table for shotgun data. 16S functional inference can be thought of as a bigger inferential leap than with shotgun, as with 16S we guess the entire genomic content and thus function based on a single sequence rather than directly inferring function from the detected genes as is the case in shotgun sequencing. In both cases, a functional analysis is limited by the validity and completeness of the functional database used to assign functional importance to the taxa or genes. The number of functional databases is currently quite limited, with KEGG, UniRef90 and MetaCyc being among the most common. Like taxonomic databases, functional databases are updated frequently and results may be affected as a consequence. In general, the functional microbiome is known to be more consistent between hosts than the taxonomic

microbiome, making it a method to reduce inter-subject variance worth consideration (Mehta et al., 2018).

1.2.4 Exploring the mesoscale

1.2.4.1 Mesoscale features

Mesoscale features of the microbiome are features that do not necessarily contain information about its smallest parts, the *microscale*, like microbial taxa, nor about the whole system, the *macroscale*, like alpha diversity, but rather contain information about patterns within *parts* of a microbiome that can be seen across samples. Mesoscale analysis focuses on identifying community-level patterns that define the ecosystem(s) under study. The mesoscale is an important object of study in theoretical ecology (Hogeweg, 2010). Here, some of the more common types of mesoscale features will be discussed.

1.2.4.1.1 Ecological Guilds

Ecological guilds are *taxonomically unrelated* but *functionally related* clusters of microbes that fulfil a certain function in the microbiome (e.g., occupy a common niche). For example, microbial communities across a wide span of environments including soil, the ocean, and the human gut can be modelled as being organised in *trophic groups* that feed on one set of substrates and subsequently pass on metabolites to another trophic group. While ecological guilds are a promising concept in microbiome science, to our knowledge there are currently no standardized pipelines or databases that can be used to detect and compare ecological guilds across cohorts and experiments. Such tools would be welcome additions to the field (Lam et al., 2018; Zhao et al., 2018).

1.2.4.1.2 Functional Modules

Functional modules are a list of curated metabolic pathways encoding for processes that are related to a specific aspect of the microbiome. As of now, there exist two classes of functional modules. *Gut-Brain modules* cover pathways that are related to gut-brain communication, such as serotonin degradation or histamine production. The complete list of Gut-Brain Modules can be accessed as a table in the supplementary files of the paper that introduced them (Valles-Colomer et al., 2019)

as well as online (<http://raeslab.org/software/gbms.html>). *Gut-Metabolic Modules* cover metabolic processes in the microbiome. Changes in Gut-Metabolic Modules can indicate a shift in the microbial metabolic environment and thereby in the fitness landscape, thus allowing for microbes with different metabolic features to thrive. The complete list of Gut-Metabolic Modules can be accessed as a table in the supplementary files of the paper that introduced them (Vieira-Silva et al., 2016) as well as on GitHub (<https://github.com/raeslab/GMMs>). Functional modules are especially useful because they can be very easy to interpret and sometimes allow for clear hypothesis development for future experiments.

1.2.5 A word on compositional data

It may seem tempting to directly treat count data, such as from the microbiome, as one would normally treat other types of measurements, perhaps converting the count data to percentages or even performing a logarithm, or square root transformation to normalize the data. Indeed, up until recently most microbiome studies were conducted in this manner. In the past few years, more and higher impact microbiome studies employing compositional data analysis (CoDa) have been published, indicating that this approach is gaining traction (Johnson et al., 2019; Valles-Colomer et al., 2019; Martino et al., 2020).

Microbiome datasets are compositional, which comes with a set of properties that, if ignored, will lead to an underpowered analysis and numerous spurious results. There are excellent reviews on CoDa in general and how it relates to the microbiome in particular that we encourage our audience to read (Aitchison, 1982; Gloor et al., 2017).

1.2.5.1 A straightforward and solid approach to account for compositionality in microbiome data

In this guidebook, we will recommend performing a centered log-ratio (CLR) transformation on the count data before performing any plotting or statistical analysis like normal, with three exceptions. First, alpha diversity should not be done on transformed data. Second, stacked bar plots should rather be generated using

counts normalized to 1 or to percentages. Third, correlating taxa or other features to each other, for example with the intent of performing a network analysis, warrants more attention and will be covered in its own paragraph.

Because microbiome count data is typically zero-inflated and, as the name implies, a centered log-ratio transformation involves taking a logarithm and, as is well-known, the logarithm of zero is undefined, the zeroes in microbiome count data must first be dealt with. Several good solutions have been proposed and in practice the exact method chosen from these solutions is of little consequence. In the chapters found in this thesis, I employ an approach derived from the ALDEx2 framework (Fernandes et al., 2013). In short, we take the median of the CLR-transformed Monte Carlo samples of the Dirichlet distribution for each sample as an estimate for what the theoretical CLR-transformed values would be. After the CLR-transformation, the values of features will have a domain of \mathbb{R} , rather than count data, which cannot be negative. From this point onwards, classical statistical approaches can be applied as normal.

1.2.5.2 Compositions and correlations between microbiome features

One of the properties of compositional data is that features are innately negatively correlated, rendering popular methods to assess correlations, like Pearson's r , Spearman's ρ or even Kendall's τ unreliable. Indeed, Karl Pearson warned against applying his namesake Pearson's correlation coefficient on compositional data (Pearson, 1897). Effective and compositionally appropriate alternatives exist. Here, we recommend the ϕ and ρ metrics found in the *propr* library in R (Quinn et al., 2017). In a nutshell, these metrics assess proportionality; if the ratio between two features remains constant through many observations, this is an indication that the two are associated (Gloor et al., 2017; Quinn et al., 2017).

1.3 Specific Aims and Hypotheses

1.3.1 General aim

In this thesis I set out to investigate what features in the microbiome are most informative in regard to gut-brain communication. Given the highly complex nature of the microbiome and the quite specific ways in which we can practically measure the microbiome (i.e. mostly metagenomics and sometimes metabolomics), finding helpful and informative features that to measure is crucial when conducting a microbiome study. There is a consensus that diversity and differential taxon abundance should be considered, but other than this, there are few concrete guidelines to follow. In order to find and apply informative metrics, we set out to investigate the interplay between the microbiome and brain and behaviour in different settings.

1.3.2 Sub-aims

The figure below shows the aspects of the microbiome-gut-brain axis that we set out to investigate to identify our metrics of interest.

1.3.2.1 Neurobehavioural phenotype

Defining experimental groups based on neurobehavioural differences is a classic approach in the microbiome-gut-brain axis field. In chapter 5.1, we investigate the differences in microbiome composition between deer mice that build large nest and those that build normal nests. A proportion of deer mice will naturally exhibit large nest building behaviour and they are used as an animal model for OCD. Indeed, large nest building deer mice have been shown to respond to SSRIs, which are commonly prescribed for OCD.

1.3.2.2 Stress

It is well known that stress affects the microbiome, though the specific taxa that are altered by stress seem to differ between studies. Stress and the microbiome represent a major aspect of this thesis and their interplay is investigated in chapters

3.1, 3.2 and most notably in 5.1, which focuses on the effects of chronic stress on the microbiome in both mice and humans.

1.3.2.3 Critical windows

When Paracelsus famously claimed that *dosis sola facit venenum* (only the dose makes the poison), he unfortunately missed that timing is also important. It is evident that timing is crucial when it comes to the microbiome. Microbiome perturbations during colonization at early life are known to have consequences for the host later in life. In chapters 3.3, 3.5, 4.2 and 4.4, we study animal models of the microbiome undergoing early life adverse events and perturbations. In a category on its own, we studied the feasibility of targeting the microbiome during adolescence to cushion the symptoms of ageing in chapter 3.1.

1.3.2.4 Diet

Diet is known to be one of the major factors shaping the microbiome. It is also known to play a role in modulating brain and behaviour. In all subchapters of chapter 3, we investigate the effects of diet on the microbiome in animal models. Furthermore, chapter 7.3 features an observational study where we follow 24 human participants during a 12 week course where they all eat from the same diet rich in unpasteurized dairy.

1.3.2.5 Perturbation

Perturbation of the microbiome is a broad term, but it captures an important aspect of my fascination with the microbiome. Here, perturbation is used to indicate alterations in the microbiome due to factors that are generally thought of as harmful, such as antibiotics or even host stress. In all subchapters of chapter 4 we investigate the dynamics of perturbation, robustness and recovery. Furthermore, we zoom in on the volatile nature of the microbiome in rest and during stress in chapter 6.1, on volatility.

1.3.2.6 Psychobiotic Intervention

A psychobiotic intervention will on first glance mean the administration of pre- or probiotics. However, it can be interpreted as any treatment that targets the microbiome composition with the goal to positively influence host mental health and

cognition. As such, I invite the reader to also think of FMT and certain dietary interventions as psychobiotic. Psychobiotic interventions are the most common theme in this thesis, but they can be found in particular in chapter 4.1 and 4.3, which feature FMT as well as 4.4, 7.2 and 7.3 which feature probiotics and unpasteurized dairy.

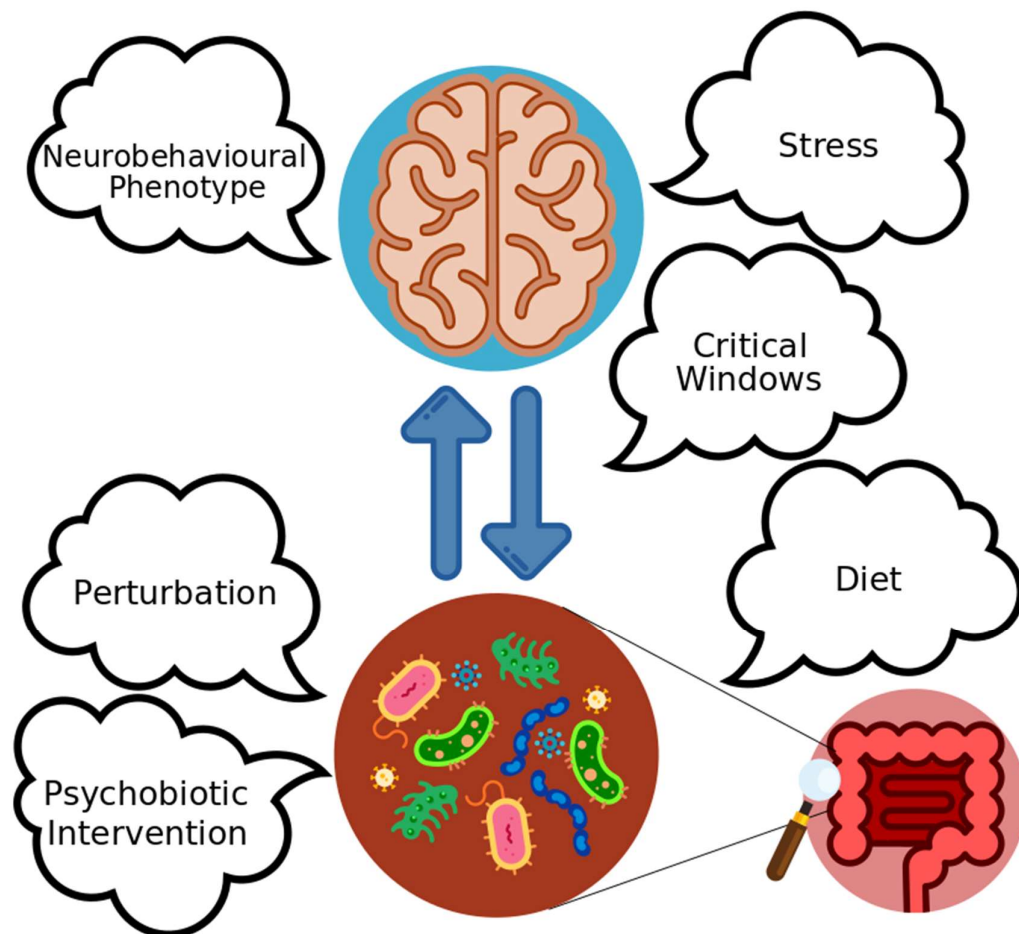


Figure 1.3-1 summarising the aspects of the microbiota-gut-brain axis that have been investigated in the chapters in this thesis.

1.4 Summary of the Presented Papers and Manuscripts by subchapter

Here, I will give a heavily abridged summary of every manuscript presented in this thesis, as to help remind the reader what the general subject and findings of a given manuscript were. For the published work, I will also provide the DOI on the title page of the subchapter. Supplementary figures and some tables have been omitted from this thesis and are all available online.

Chapter 2 Targeting the Microbiome-Gut-Brain Axis with Diet

2.1 Mid-Life Microbiota Crises: Middle Age is Associated with Pervasive Neuroimmune Alterations that are Reversed by Targeting the Gut Microbiome

Ageing comes with a host of physiological and behavioural changes including cognitive decline. FOS-Inulin supplementation counteracted the neuroinflammatory phenotype normally found in middle-aged mice after stress. Notably, both ageing and prebiotics supplementation showed their own signature in the microbiome.

2.2 Preventing adolescent stress-induced cognitive and microbiome changes by diet

Stress impacts the host in many ways, including behaviour and the microbiome. By supplementing rats with a diet rich in omega-3 fatty acids and vitamin A, these changes in both the microbiome and in behaviour were decreased. Notably, the effect of the enriched diet remained visible in adulthood.

2.3 Polyphenols selectively reverse early-life stress-induced behavioural, neurochemical and microbiota changes in the rat

Early life stress is known to promote anxiety and depressive-like behaviour later in life. Polyphenol supplementation rescued these early life stress effects and changed the microbiome, including its functional potential.

2.4 Prebiotic administration modulates gut microbiota and faecal short-chain fatty acid concentrations but does not prevent chronic intermittent hypoxia-induced apnoea and hypertension in adult rats

Hypoxia can promote cardiorespiratory morbidity and also affects the microbiome. Prebiotic (FOS/GOS) supplementation did not rescue cardiorespiratory effects of hypoxia, but affected the microbiome strongly, both in terms of taxonomy as in function.

2.5 Adolescent Dietary Manipulations Differentially Affect Gut Microbiota Composition and Amygdala Neuroimmune Gene Expression in Male Mice in Adulthood

Poor diets such as high fat or even cafeteria diet in adolescence affect the host during adulthood in many ways, including in terms of neuroimmunity. In adult mice, the effects of these diets are still visible in the microbiome, both in terms of differential taxa abundance as in beta diversity.

Chapter 3 Targeting the Microbiome with Perturbations and Psychobiotics

3.1 Microbiota from Young Mice Selectively Counteracts the Effects of Aging Across the Microbiome-Gut-Immune-Brain Axis

In addition to impacting host physiology, immune system brain and behaviour, ageing impacts the host microbiome in specific manners. We show that Faecal Microbiota Transplantation (FMT) from young to aged mice counteracts some, but not all, of these age-induced changes.

3.2 Enduring neurobehavioral effects induced by microbiota depletion during the adolescent period

Antibiotics perturbation of the microbiome during adolescence can have long lasting consequences with regards to brain and behaviour. While the effects of antibiotics were no longer detectable in the microbiome in adulthood, a behavioural phenotype persists. This suggests some sort of critical window where adolescent mice are susceptible to antibiotics perturbation.

3.3 Strategies for effective gut microbiota recovery after chronic broad-spectrum antibiotic administration in adult male rats.

The way in which the microbiome restores itself after antibiotics depletion remains unclear. Strikingly, the genus *Blautia* was only able to colonize after ABX perturbation followed by prebiotics supplementation, but not after just ABX or just prebiotics alone. This colonization of *Blautia* was linked back to changes in functional capacity.

3.4 Enduring behavioral effects induced by birth by caesarean section in the mouse

C-section alters the starting state of the microbiome, as the offspring is never in contact with the birth canal, where microbiome colonisation is thought to typically take place. In early life, the microbiome of mice delivered by c-section is clearly different from that of naturally born animals. In adulthood, these changes can no longer be detected in the microbiome, but a signature remains in brain gene expression and in behaviour.

Chapter 4

4.1 Natural compulsive-like behaviour in the deer mouse (*Peromyscus maniculatus bairdii*) is associated with altered gut microbiota composition

The deer mouse builds nests. Naturally, a proportion of deer mice builds much larger nests than the rest. The deer mouse is used as a model organism for OCD. We find that microbiome composition, but not any specific microbial genera, is associated with nest builder status. This in turn has implications for OCD being partially modulated by the microbiome.

Chapter 5

5.1 Volatility as a Concept to Understand the Impact of Stress on the Microbiome

We find that degree of change in the microbiome after a stressor is correlated with severity of the stress response, both in mice and in students undergoing academic stress. Furthermore, we find that, while which genera are altered due to stress is dependent on the cohort, functional changes are consistent between cohorts and even between mice and humans.

Chapter 6 Human Microbiome-Gut-Brain Axis Studies

6.1 A specific dietary fibre supplementation improves cognitive performance—an exploratory randomised, placebo-controlled, crossover study

The prebiotic polydextrose improved scores in cognitive tasks. In the microbiome, it specifically increased relative abundance of the genus *Ruminiclostridium* but did not alter the microbiome composition detectably on a beta diversity level.

6.2 Bifidobacterium longum Counters the Effects of Obesity: Partial Successful Translation from Rodent to Human

In this translational study, the probiotic *Bifidobacterium longum* APC1472 was shown to have anti-obesity effects in mice but these were not replicated in humans. In a longitudinal cohort of obese and overweight (human) participants, recipients showed an improvement in other measurements, including higher ghrelin levels and reduced cortisol awakening response.

6.3 Recipe for a Healthy Gut: Intake of Unpasteurised Milk Is Associated with Increased Lactobacillus Abundance in the Human Gut Microbiome

After a 12-week residential cookery course where students have an increased intake in unpasteurized dairy, we find that milk-fermenting bacteria are strongly increased in the gut microbiome. We also find an increase in neuroactive gut-brain modules, as well as an overall increase in functional diversity.

Chapter 2 Targeting the Microbiome- Gut-Brain Axis with Diet

2.1 Mid-life microbiota crises: middle age is associated with pervasive neuroimmune alterations that are reversed by targeting the gut microbiome

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2.1.1 Abstract

Male middle age is a transitional period where many physiological and psychological changes occur leading to cognitive and behavioural alterations, and a deterioration of brain function. However, the mechanisms underpinning such changes are unclear. The gut microbiome has been implicated as a key mediator in the communication between the gut and the brain, and in the regulation of brain homeostasis including brain immune cell function. Thus, we tested whether targeting the gut microbiome by prebiotic supplementation, may alter microglia activation and brain function in ageing. Male young adult (eight weeks) and middle-aged C57BL/6J mice (ten months) received diet enriched with a prebiotic (10% oligofructose-enriched inulin (FOS-Inulin)) or control chow for 14 weeks. Prebiotic supplementation differentially altered the gut microbiota profile in young and middle-aged mice with changes correlating with faecal metabolites. Functionally, this translated into a reversal of stress-induced immune priming in middle-aged mice. In addition, a reduction in ageing-induced infiltration of Ly-6C^{hi}-monocytes into the brain coupled with a reversal in ageing-related increases in a subset of activated microglia (Ly-6C⁺) was observed. Taken together, these data highlight a potential pathway by which targeting the gut microbiome with prebiotics can modulate the peripheral immune response and alter neuroinflammation in middle age. Our data highlight a novel strategy for the amelioration of age-related neuroinflammatory pathologies and brain function.

2.1.2 Introduction

We have trillions of microbes in our gastrointestinal tract, and a growing body of evidence supports a role for them in maintaining health across the lifespan (Lynch and Pedersen, 2016; Fung et al., 2017; Miquel et al., 2018). Indeed, microbiota has been implicated as a key mediator in the communication between the gut and the brain and regulating brain homeostasis. Diet has been shown to be one of the most important factors in modifying the gut microbiota composition (David et al., 2014; Sandhu et al., 2017). However, the ability of nutritional interventions that target the microbiome to alter brain function has not received much attention (Donovan, 2017; Sandhu et al., 2017; Miquel et al., 2018).

Ageing is defined as a process involving slow deterioration of various homeostatic functions throughout the lifespan. Middle age in particular is a life period where many physiological and psychological changes occur, leading to first cognitive impairments and behavioural alterations, and a deterioration of brain function (Francia et al., 2006; Ennaceur et al., 2008; Duarte et al., 2014; Bensalem et al., 2016; Shoji et al., 2016). In rodents, increased anxiety-like behaviour occurs in middle-age (Ennaceur et al., 2008; Shoji et al., 2016). A few studies reported cognitive decline in middle-aged rodents (Bensalem et al., 2016; Shoji et al., 2016), with variable definitions of “middle-age” highlighting the need for greater specification (Prenderville et al., 2015). Moreover, the levels of neurotransmitters (Duarte et al., 2014) and neurotrophins (Francia et al., 2006) were shown to decline with age, which may possibly contribute to altered behaviour and brain homeostasis.

Increased age is associated with a shift towards a pro-inflammatory state and inflammageing (Sparkman and Johnson, 2008; Franceschi et al., 2017). This, in turn, can make the ageing brain more vulnerable to various intrinsic and extrinsic disruptive effects including stress, disease and infection (Norden and Godbout, 2013; Prenderville et al., 2015). Moreover, this vulnerability may result in cognitive alterations (Miquel et al., 2018). However, it remains unclear to what extent an

altered brain immune system can contribute to alterations in cognitive functions in middle-aged subjects.

Microglia are the major immune cells in the brain and have been shown to be a key player in neuropsychological and neurodegenerative conditions (Tay et al., 2017; Hickman et al., 2018). Increased activation of microglia in the aged brain has been suggested to be indicative of enhanced inflammation and heightened reactivity in the rodent and the human brain (Perry et al., 2003; Streit et al., 2004; Sparkman and Johnson, 2008). Following an immune stimulus, which is exaggerated in ageing, microglia are referred to as “primed” due to their rapid induction and increased cytokine release upon activation (Perry et al., 2003; Sparkman and Johnson, 2008). Microglia are specialised cells continuously monitoring their environment (Nimmerjahn et al., 2005) and can sense changes in the brain’s milieu (Hickman et al., 2013). In addition, microglia play a crucial role in synaptic plasticity, brain function and cognition across the lifespan (Tay et al., 2017).

Numerous studies have shown shifts in the composition of the intestinal microbiota with age in rodent models (Scott et al., 2017; van der Lugt et al., 2018) and in humans, including extreme ageing (Biagi et al., 2010; Biagi et al., 2016). Previous research utilizing pre-clinical models implicated a role of microbiota from aged mice in driving systemic immunity (Fransen et al., 2017; Thevaranjan et al., 2017). However, the effect on neuro-immunity and subsequent brain function and behaviour remains unaddressed. Interestingly, the transfer of gut microbiota from young-to-aged might influence healthy ageing as shown in the short-lived killifish model, which exhibited an increase in lifespan and motor behaviour (Smith et al., 2017). It has been shown that the administration of prebiotics (a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)) results in an increase in the number of beneficial intestinal bacterial species with a reduction in systemic inflammation in rodents (Burokas et al., 2017) and humans (Schiffrin et al., 2007; Vulevic et al., 2008). Moreover, that prebiotics may alter microglia activation

in the aged brain (Matt et al., 2018) which would have important implications for the healthcare system. It however remains unclear what is driving these changes and what is the impact on brain function and behaviour, i.e. cognitive behaviour. Therapeutic interventions aim to delay ageing, decrease pro-ageing factors, reduce microglia activation and ultimately improve cognition during ageing.

We hypothesise that there is a dysregulation in the communication between the gut microbiota and the brain during middle age, which is critical in mediating age-related functional decline. Thus, targeting the gut microbiota with prebiotics may alter microglia activation state and brain function in ageing. To this end, we hypothesised that targeting the gut microbiome by dietary intervention with a complex short- and long-chain prebiotic, oligofructose-enriched inulin (FOS-Inulin), would have selective effects on (neuro-) immune profile and behaviour in middle-aged male compared to young adult C57BL/6J mice.

2.1.3 Methods

2.1.3.1 Animals

Male young adult C57BL/6J mice (n = 60; Harlan, Cambridgeshire, UK; 2 months) and middle-aged C57BL/6J mice (n = 48; 10 months) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork (B100/3774). Animals were habituated to the animal facility for two weeks before experiments started and kept under a 12-hour light/dark cycle, with a temperature of 21 ± 1 °C and humidity of $55 \pm 10\%$. Food and water were given *ad libitum*.

2.1.3.2 Prebiotic administration

Mice received chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% Oligofructose-enriched inulin (FOS-Inulin: mixture of $92 \pm 2\%$ Inulin and $8 \pm 2\%$ Fructooligosaccharide, Orafti®Synergy1; BNEO-Orafti N.V., Tienen, Belgium) or control chow for 3.5 weeks (microglia cohort) and 14 weeks (behavioural cohort). The dosage of FOS-Inulin supplementation was chosen based on previous studies in rodents (Messaoudi et al., 2005; Rault-Nania et al., 2006; Rozan et al., 2008). Duration of prebiotic intervention was chosen according to previous studies in rodents showing effects on brain and behaviour (Savignac et al., 2015; Burokas et al., 2017).

2.1.3.3 Study design and experimental timeline

Two separate cohorts of animals were used (see Supplementary Figure S1).

Cohort one investigated the effects of FOS-Inulin on behaviour including cognitive (spontaneous alternation behaviour, Morris water maze, fear conditioning), anxiety-like (open field, elevated-plus maze, marble burying), social (three-chamber social interaction test) and depressive-like behaviour (forced swim test). Following a three-week lead-in of diet, mice (n=8-10 per group) underwent behavioural assessment while continuing dietary supplementation for a total of 14 weeks. In addition,

peripheral immune cell activation (pre-/post stress) was assessed in blood by using flow cytometry. To correlate the changes in behaviour with specific neuroimmune targets, we subsequently analysed targets in the brain at the end of the study.

To characterize the neuroimmune status in the brain at a time point that coincided with that before animals were tested behaviourally, cohort two (young adult: n=14-16, middle-aged: n=8-10) investigated if a dietary lead-in phase of 3.5 weeks with FOS-Inulin can alter monocyte infiltration and subsequent microglia activation in the brain, key mediators influencing cognition and anxiety-like behaviour.

2.1.3.4 Behaviour

2.1.3.4.1 Spontaneous alternation in the Y-Maze

Spontaneous alternation behaviour in the Y-maze tests hippocampal-dependent spatial memory and exploration exploratory activity and was carried out as previously described (Scott et al., 2017). Behaviour was assessed for five minutes.

2.1.3.4.2 Morris water maze

The Morris water maze represents a robust and reliable test for spatial learning that strongly correlates with hippocampal synaptic plasticity (Vorhees and Williams, 2006). Briefly, mice were trained over five days (four trials per day, two minutes each) to spatially locate the submerged platform. On day six, the platform was removed and a probe trial lasting 30s conducted.

2.1.3.4.3 Fear conditioning

Fear conditioning was conducted as previously described (Izquierdo et al., 2006), over three consecutive days (day 1: conditioning by three pairings with variable inter-pairing interval; day 2: conditioned stimulus recall and extinction in a novel context; day 3: context recall).

2.1.3.4.4 Open field

The open field is a widely used test to assess approach-avoidance behaviour, locomotor activity and the behavioural response to a novel context, and was conducted as previously described (Burokas et al., 2017). Briefly, a test mouse was placed into an open arena with 60 lux lighting and allowed to explore the context for ten minutes.

2.1.3.4.5 Marble burying test

The marble burying test assesses compulsive, repetitive and anxiety-like behaviour, and was conducted as previously described (Burokas et al., 2017). Briefly, mice were tested for 30 min and the number of buried marbles was recorded.

2.1.3.4.6 Elevated-plus maze

The Elevated-plus Maze test was used to assess anxiety-like behaviour and was conducted as previously described (Burokas et al., 2017). Mice were allowed to explore the maze for five minutes; the time spent in the open arms, as well as number of entries into the open arms was analysed.

2.1.3.4.7 Three-chamber social interaction test

Sociability and social novelty were assessed in a three-chamber apparatus as previously described (Desbonnet et al., 2012). The test consists of three sequential ten minute trials: (1) habituation; (2) sociability (the analysis of time a test mouse spends in the chamber with the conspecific mouse or with the object). (3) social novelty preference (the analysis of time a test mouse spends in the chamber with the novel or in the chamber with the familiar mouse).

2.1.3.4.8 Forced swim test

The forced swim test was used to assess depressive-like or despair-like behaviour (Porsolt et al., 1977; Cryan and Mombereau, 2004). Mice were individually placed in

a transparent glass cylinder for six minutes. Time spent immobile was defined as no movements apart from breathing and considered as depressive-like behaviour. Behaviour was analysed during the last 4 minutes of the test which represents the most common protocol to use in analysing FST in the mouse and accounts for the fact that most mice struggle heavily during the first two minutes as they habituate to the water situation (Porsolt et al., 1977; Cryan and Mombereau, 2004).

2.1.3.5 Plasma collection and corticosterone analysis

To investigate the endocrine and immune response to stress, we collected blood samples prior to and following the forced swim test session. Approximately 60 µl of blood per mouse were collected by tail tipping using Lithium-Heparin-coated capillaries (Sigma-Aldrich, St. Louis, Missouri, United States). Blood was centrifuged at 3500 g at 4 °C for 15 min. Plasma was aspirated and stored at –80°C. Blood was taken immediately before the forced swim test (baseline), as well as 15 min, 45 min and 120 min after the baseline. Baseline samples and samples at 120 min post-stress time point were used for flow cytometry (see 2.7).

Plasma corticosterone levels were measured in duplicates by ELISA (ENZO Corticosterone ELISA, Enzo Life Sciences, Exeter, UK) as previously described (Scott et al., 2017). Data were expressed in ng/ml. Only data derived from duplicates with < 15% coefficient of variation (CV) were included in the analysis.

2.1.3.6 Blood stimulation cytokine assay

To assess if a prebiotic-enriched diet alters systemic immunity, 100 µl of trunk blood was obtained at the end of the study using Lithium-Heparin-coated tubes (Greiner Bio-One, Kremsmünster, Austria). Blood cells from each mouse were stimulated with lipopolysaccharide (LPS-2 µg/ml) or Concanavalin A (ConA-2.5 µg/ml) for 24 h or left unstimulated as control. Following 24 h-incubation, samples were taken and stored at -80°C. The levels of secreted IL-1β, IL-4, IL-6, IL-10, TNFα and CXCL1 were analysed with the Proinflammatory Panel 1 (mouse) V-PLEX Kit and the MESO QuickPlex SQ 120, SECTOR Imager 2400 (Meso Scale Discovery, Maryland, USA). Only data derived

from duplicates with < 15% CV were included in the analysis. Concentrations of cytokines were expressed in pg/ml.

2.1.3.7 Flow cytometry

To assess stress-induced immune priming, blood was collected from young adult and middle-aged mice by tail tipping (60 µl) at baseline and 120 min after acute stress (cohort one). Staining was performed using CD11b-VioBright FITC, Ly-6C-PE, LY-6G-PerCP-Vio700 and MHC-II-PE (all Miltenyi Biotec, Bergisch Gladbach, Germany) to assess inflammatory monocytes (CD11b⁺, SSC^{low}, LY-6C^{hi}) and MHC-II⁺-neutrophils (CD11b⁺, LY-6G⁺, MHC-II⁺). Inflammatory monocyte and MHC-II⁺-neutrophil counts were normalized to the amount of peripheral blood mononuclear cell (PBMC). Gating strategy is depicted in Supplementary Figure S2a.

Cohort two investigated if the diet lead-in phase with FOS-Inulin modulates monocyte infiltration and subsequent microglia activation in the brain. Following perfusion with ice-cold PBS for five min, brains were carefully dissected, enzymatically digested using the neural dissociation kit (P), followed by incubation in myelin-removal beads and magnetic separation using LS columns (Miltenyi Biotec). Cells were stained using CD11b-Viobright FITC, CD45-APC and Ly-6C-PE (all Miltenyi Biotec). Gating strategy is depicted in Supplementary Figure S2b. Monocyte counts were normalized to CD11b⁺ cells, microglia to CD11b⁺, CD45^{low}.

2.1.3.8 Analysis of gene expression levels in the brain tissues (RT-qPCR)

To assess gene expression brain areas associated with cognition, the right hemisphere of both, the hippocampus and the prefrontal cortex were used (Schellekens et al., 2012). Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion, Life technologies, Waltham, MA, US), followed by DNase treatment using the TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's instructions. RNA was quantified using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied Biosystems, Warrington, UK). *Ccl2* and *Tnf* genes were amplified with probes designed by Integrated DNA Technologies (Coralville, IA, US) (Table S1). PCR was run in triplicates on a LightCycler®480 (Roche). Data were analysed with the comparative cycle threshold (Ct) method. Data were normalized using *Actb* as endogenous control and transformed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). We confirmed beforehand that the housekeeper *Actb* is neither changed by age nor by prebiotic treatment.

2.1.3.9 Caecal microbiota composition (16S rRNA gene sequencing) and short-chain fatty acid analysis

Caecum was harvested, snap frozen and stored at -80°C prior to the analysis. DNA from caecal content was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step, as previously described . The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol. Samples were sequenced at Teagasc Sequencing Facility on the Illumina MiSeq platform using a 2 × 250 bp kit.

FLASH was used to assemble paired-end reads. Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (version 1.9.0). Denoising, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit) (Edgar, 2010). OTUs were aligned using PyNAST (and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Alpha and beta diversities were generated in QIIME (Caporaso et al., 2010).

Short chain fatty acids (SCFAs) were measured by gas chromatography, using a Varian 3500 GC flame-ionization system fitted with a ZB-FFAP column as previously described (de Wouw et al., 2018).

2.1.3.10 Metabolomics from faecal water

Faecal pellets were collected at the end of cohort one. Faecal material were freshly collected with sterilized tools to ensure no cross contamination within a time-window of 10 minutes' maximum to ensure least oxygen exposure of the faeces as possible. Subsequently, pellets were directly snap freeze to ensure optimal DNA integrity. Faecal water was prepared by homogenising faecal samples (20-50 mg) with 4x wt/volume sterile PBS followed by vortexing for 20 minutes. Samples were centrifuged at 16000 g for 30 minutes; the supernatant was transferred in a new 2 mL micro centrifuge tube and centrifuged for further 30 minutes. This step was repeated one more time before filtering the supernatant through Costar Spin-X centrifuge filters 0.2 μ M at 10000 g. Faecal water samples were stored at -20°C.

Subsequently, samples were derivatized with methyl chloroformate as previously described (Smart et al., 2010) and processed by MS-Omics (Copenhagen, Denmark) using Gas Chromatography – Mass Spectrometry (GC-MS). Raw data was converted to netCDF format using ChemStation (Agilent technologies) and processed in Matlab R2014b (Mathworks, Inc., Natick, MA, USA) using the PARADiSe software described by (Johnsen et al., 2017).

2.1.3.11 Statistical analysis

Statistical analyses were conducted using SPSS 24 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analysed for normality using the Shapiro-Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with Kruskal-Wallis test followed by post-hoc Dunn's, and are depicted as median with inter-quartile ranges (IQR) and min/max values as error bars. Parametric data were analysed using two-way analysis of variance (ANOVA) post-hoc Holm-Sidak, and are shown as mean \pm SEM. Changes in corticosterone response, Morris Water Maze learning and fear conditioning were analysed using two-way-repeated measurement (RM)-ANOVA post-hoc Sidak. Correlation analyses were performed using Spearman correlations for non-parametric data. Outliers were excluded using the Grubbs test. Statistical significance was set at $p \leq 0.05$.

Statistical analysis of microbiota data was performed in an R software environment. For Principal Component Analysis (PCA), Permutational multivariate analysis of variance (PERMANOVA) was used to identify relationships of significance between variables the Adonis function from the vegan package on Aitchison distance matrices calculated with the ALDEx2 package. ALDEx2 was also used to calculate pairwise differential abundance. Hierarchical All-against-All significance (HALLA) was used to investigate between-dataset covariance. For all tests, a Benjamini-Hochberg post-hoc test was performed to correct for multiple comparisons with a conservative q -value of 0.2 as critical value.

2.1.4 Results

2.1.4.1 Prebiotic supplementation reversed stress-induced immune priming in ageing

To assess if ageing triggers stress-induced immune priming in middle-aged mice, and whether age-associated changes are alleviated following prebiotic supplementation, mice were exposed to an acute stress (forced swim test), and blood samples were taken at baseline and 2 hours after stress exposure. We focused on neutrophils, which act as the first responders to any immune challenge and can trigger adaptive immunity, including T-cell priming via expression of major histocompatibility complexes (MHC), a classical activation marker.

In middle-aged mice, acute stress caused a long-lasting increase of the MHC-II+ neutrophil population ($p=0.0280$, Kruskal-Wallis post-hoc Dunn's; Figure 2.1-1a); the response being absent in young adult animals. Strikingly, prebiotic supplementation prevented the development of the age-associated phenotype and restored the levels of MHC-II+ neutrophils in stressed aged animals to young levels ($p=0.011$).

Since acute stress is known to affect peripheral innate immunity through corticosterone (Dhabhar et al., 2012), we investigated whether these changes in neutrophil activation status were associated with altered stress axis activity. For this, we measured plasma release of corticosterone (as an indicator of endocrine reactivity to stress) in the same animals, prior to and at different time points following the forced swim stress exposure. Two-way RM-ANOVA revealed an overall effect of age on the corticosterone response ($F_{(1, 28)} = 10.825$, $p=0.003$; Figure 2.1-1b). In particular, middle-aged mice exhibited lower plasma corticosterone levels at baseline ($F_{(1, 29)} = 16.68$, $p<0.001$, Figure 2.1-1c) and at T15 ($F_{(1, 34)} = 24.65$, $p<0.001$). Calculation of area-under-the-curve (AUC) for corticosterone response confirmed that middle-aged mice exhibited a blunted stress axis reactivity ($F_{(1, 28)} = 5.207$, $p=0.03$, Figure 2.1-1d). However, we did not observe any modulation on corticosterone response neither at baseline nor following stress in neither young adult nor middle-aged mice by prebiotic supplementation suggesting that the changes in peripheral innate immunity are not mediated by corticosterone.

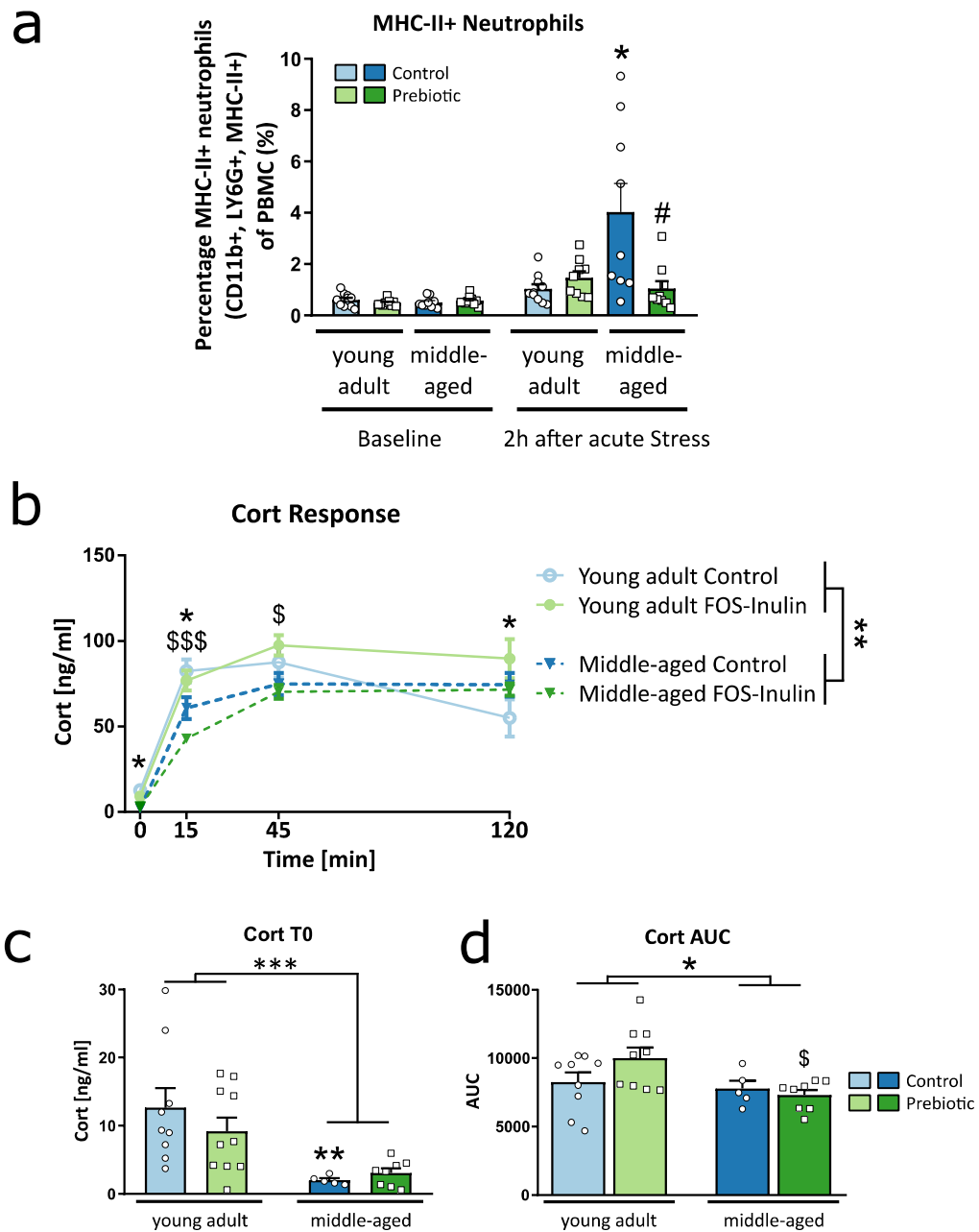


Figure 2.1-1. Prebiotic supplementation reversed stress-induced immune priming in middle-aged mice. (a) MHC-II+ neutrophils at baseline and 2h after acute stress. **(b)** Plasma Corticosterone (Cort) response curve at baseline, immediately before exposure to acute stress, and 15, 45 and 120 min after exposure to acute stress. **(c)** Plasma corticosterone at baseline. **(d)** Area-under-the-curve (AUC) of corticosterone response. Mean \pm SEM. $n = 8-10$. vs. control young adult * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control middle-aged # $p < 0.05$, ## $p < 0.01$, vs. prebiotic middle-aged vs prebiotic adult \$ $p < 0.05$.

2.1.4.2 Effect of prebiotic supplementation on systemic inflammation and immune cell priming

To investigate if systemic inflammation and immune cell priming is altered in middle-aged mice and counteracted by prebiotic supplementation, whole blood was taken after 14 weeks of prebiotic intervention and stimulated with LPS or ConA. Following ConA-stimulation, middle-aged control mice exhibited a trend towards increased IL-1 β and IL-10 cytokine release ($p=0.089$ and $p=0.069$, respectively, Supplementary Figure S3b+e), while prebiotic-treated middle-aged mice showed similar levels as in young controls. Moreover, prebiotic supplementation decreased TNF α in middle-aged mice following ConA-stimulation ($p=0.014$, Kruskal-Wallis post-hoc Dunn's; S3a). No changes were observed at baseline or in response to LPS stimulation.

2.1.4.3 Pervasive neuroimmune alterations in middle-aged mice were alleviated by prebiotic supplementation

Given the decline of cognitive function in middle-aged mice (Prenderville et al., 2015; Shoji et al., 2016), we investigated whether the middle-aged brain is more vulnerable to peripheral immune cell trafficking and subsequent microglial activation, and this status can be targeted by prebiotic supplementation, we investigated brain immunity by flow cytometry. Two-way ANOVA revealed an effect of age ($F_{(1, 41)} = 11.94$, $p=0.001$; Figure 2.1-2a) and prebiotic treatment ($F_{(1, 41)} = 7.88$, $p=0.008$) as well as an interaction of both ($F_{(1, 41)} = 6.01$, $p=0.019$) on trafficking of inflammatory monocytes (Ly-6C^{hi}) into the brain. Specifically, middle-aged control mice showed an increase in Ly-6C^{hi} monocytes compared to young controls ($p<0.001$), which was alleviated by prebiotic supplementation ($p=0.007$). We in addition investigated if these changes in infiltrating monocytes are also systemically reflected in the blood. We didn't find any differences (see Supplementary Figure S7) suggesting that the brain becomes particularly vulnerable in middle-aged mice as inflammatory monocytes traffic to inflamed tissue. Furthermore, we investigated whether the observed increase in monocyte trafficking was associated with microglia activation. Two-way ANOVA revealed an effect not only of age ($F_{(1, 43)} = 10.75$, $p=0.002$; Figure 2.1-2b), but also prebiotic treatment ($F_{(1, 43)} = 10.95$, $p=0.002$) and an interaction of both ($F_{(1, 43)} =$

13.81, $p < 0.001$) on Ly-6C⁺ microglia. Middle-aged controls showed a higher percentage of Ly-6C⁺ microglia compared to young controls ($p < 0.001$), which was reversed by prebiotic supplementation to young control levels ($p < 0.001$).

In agreement with these findings, the gene expression of *Ccl2* and *Tnf* were up-regulated in the hippocampus of middle-aged mice ($F_{(1, 35)} = 13.60$, $F_{(1, 35)} = 15.79$, $p < 0.001$; Figure 2.1-2c-d). *Ccl2* and *Tnf* encode for pro-inflammatory cytokines which are secreted from activated microglia and associated with monocyte infiltration. This supports the observation of microglia activation in the middle-aged brain, including the hippocampus, a key region controlling learning and memory. In contrast, both, *Ccl2* and *Tnf*, were not found to be upregulated in middle-aged mice following prebiotic supplementation. Furthermore, we investigated this phenomenon in another cognition-related brain region, the prefrontal cortex. In contrast to the hippocampus, no effect of age or prebiotic supplementation on *Ccl2* and *Tnf* gene expression was found (Supplementary Figure S4), suggesting a non-universal effect of prebiotic supplementation on cytokine expression across brain regions.

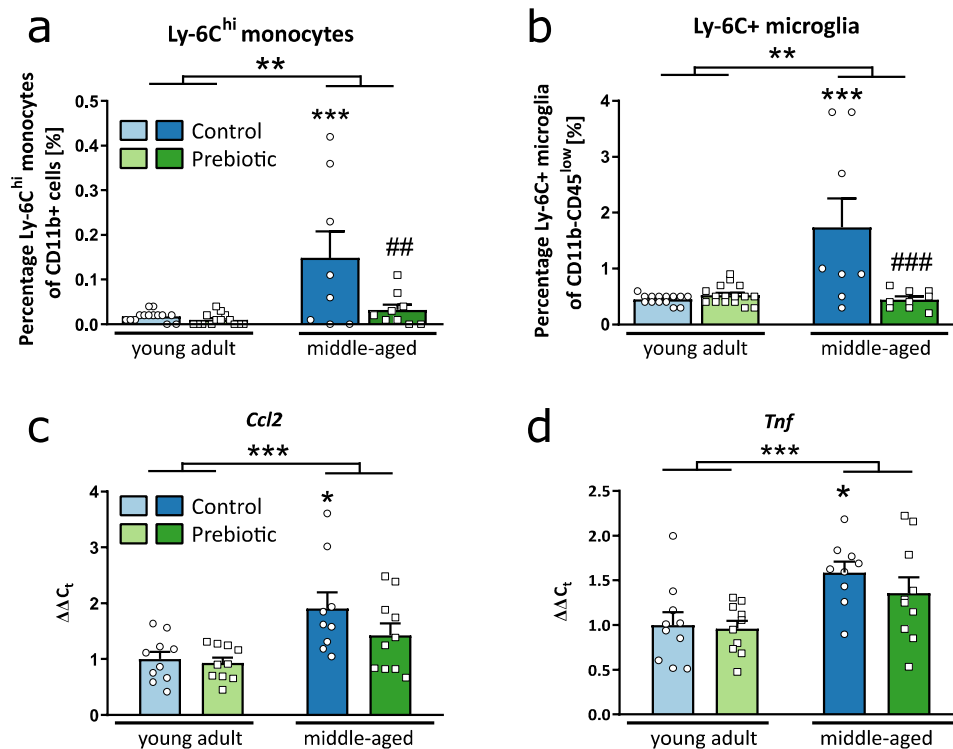


Figure 2.1-2. Middle-aged mice exhibited elevated infiltration of Ly-6C^{hi} monocytes into the brain and increased microglia activation; the phenotype was reversed by prebiotic supplementation. (a) Monocyte infiltration in the brain. (b) Microglia expression pattern in the brain. (c-d) Pro-inflammatory cytokine expression in the hippocampus. Mean \pm SEM. $n = 7-16$ (Flow Cytometry), $n = 9-10$ (Gene expression Hippocampus). vs. control young adult * $p < 0.05$, *** $p < 0.001$, vs. control middle-aged ## $p < 0.01$, ### $p < 0.001$.

2.1.4.4 Prebiotic intervention improved learning and reduced anxiety-like behaviour in young adult mice

To assess if prebiotic intervention improves spatial learning and memory, mice were trained over five consecutive days to find a hidden platform in the Morris water maze (MWM). Middle-aged mice displayed an impairment in learning ($F_{(1, 35)} = 8.653$, $p=0.006$; Figure 2.1-3a). However, prebiotic supplementation modulated learning ($F_{(1, 35)} = 10.252$, $p=0.003$), the improvement was only evident in young adult mice ($F_{(1, 18)} = 10.897$, $p=0.004$). We did not identify an interaction ($F_{(1, 35)} = 2.073$, $p=0.159$) suggesting that the prebiotic effects were specific to young adult mice. Although, the average between day four to five is visually different, both days are not statistically different from each other ($p=0.19$) and mostly explained by a much greater variation compared to day four. Similarly, area-under-the-curve (AUC) analysis confirmed the improved learning in prebiotic-treated young mice ($p=0.005$). Both, age ($F_{(1, 34)} = 13.10$, $p=0.001$) and prebiotic supplementation ($F_{(1, 34)} = 12.89$, $p=0.001$) had a modulatory impact on spatial learning. To assess spatial long-term memory, a probe trial was performed on day six. A trend towards decreased time spent in the target quadrant with age ($F_{(1, 35)} = 3.442$, $p=0.072$) was found, however, no improvement by prebiotic supplementation was observed (Figure 2.1-3a). Neither age or prebiotic exposure affected swim speed, or total distance respectively (data not shown).

We further tested the effect on short-term memory by assessing spontaneous alternation behaviour in the Y-maze. Middle-aged mice showed a decrease in spontaneous alternations ($F_{(1, 35)} = 10.66$, $p=0.003$) and total number of alternations ($F_{(1, 35)} = 7.986$, $p=0.008$; Figure 2.1-3b) suggesting impairments in short-term memory.

Next, we tested if prebiotic supplementation can modulate amygdala-dependent learning and memory. For this, mice were tested in a fear conditioning task (Figure 3c). On day one, mice were conditioned to three cued-shock pairings with a variable inter-pairing interval. Middle-aged mice displayed an impaired acquisition ($F_{(1, 36)} = 4.842$, $p=0.034$, Figure 2.1-3c). 24h later, CS recall and extinction learning were assessed. Middle-aged mice showed increased freezing during habituation to the new context ($F_{(1, 35)} = 6.702$, $p=0.014$) suggesting anxiety-like behaviour. Although statistically not significant, the changes in extinction in the prebiotic-treated young adult mice compared to the other groups are explained by the less freezing across the cue-shock pairings during acquisition. Similarly, to deficiencies in acquisition, middle-aged mice showed impairments in extinction learning ($F_{(1, 36)} = 4.898$, $p=0.034$). In contrast, no impact of age nor of prebiotic supplementation on context recall was found (Figure 2.1-3c).

Next, we analysed anxiety-like behaviour in the elevated plus maze and the open field, as changes in anxiety levels are known to affect cognitive performance. Overall, middle-aged mice displayed increased anxiety-like behaviour, as shown by less time spent in the aversive open arms of the elevated plus maze ($F_{(1,33)} = 18.31$ $p<0.001$; Figure 2.1-3d), the central zone of the open field arena ($F_{(1, 34)} = 7.337$, $p=0.011$; Figure 2.1-3e) as well as decreased number of centre visits ($F_{(1, 34)} = 14.69$, $p<0.001$). The locomotor activity was also marginally reduced in middle-aged mice ($F_{(1,33)} = 4.538$, $p=0.041$; Figure 2.1-3c). Prebiotic supplementation did not affect anxiety levels in middle-aged mice. However, a significant increase in the time spent in the open arms of the elevated plus maze was observed in young adult prebiotic-treated mice ($p=0.027$). This suggests that prebiotic supplementation did have an anxiolytic-like effect, but in young animals only. The observed changes in anxiety-like behaviour, i.e. increased anxiety levels in aged mice and selective anxiolytic effect in prebiotic-treated young mice, had a similar pattern seen in the spatial recognition in the MWM. This suggests that impaired cognitive performance in middle-aged mice, as well as improved learning of prebiotic-treated young adults could be partially mediated by changes in anxiety levels.

Interestingly, learning performance in the Morris water maze correlated with the relative abundance of the *Verrucomicrobiaceae* family ($r_{(38)} = -0.369$, $p=0.023$; Figure 2.1-3f); wherein the association is mainly driven by *Akkermansia*, the predominant genus within the *Verrucomicrobiaceae* ($r_{(38)} = -0.323$, $p=0.048$; Figure 2.1-3f). Moreover, we identified a significant correlation between hippocampal *Ccl2* expression (as a readout of microglia activation linked to monocyte trafficking) and learning performance (AUC) in the MWM task ($r_{(39)} = 0.349$, $p=0.03$; Figure 2.1-3g). To emphasize these correlations further, we indicated which data points relates to which group indicating that prebiotics drive these associations.

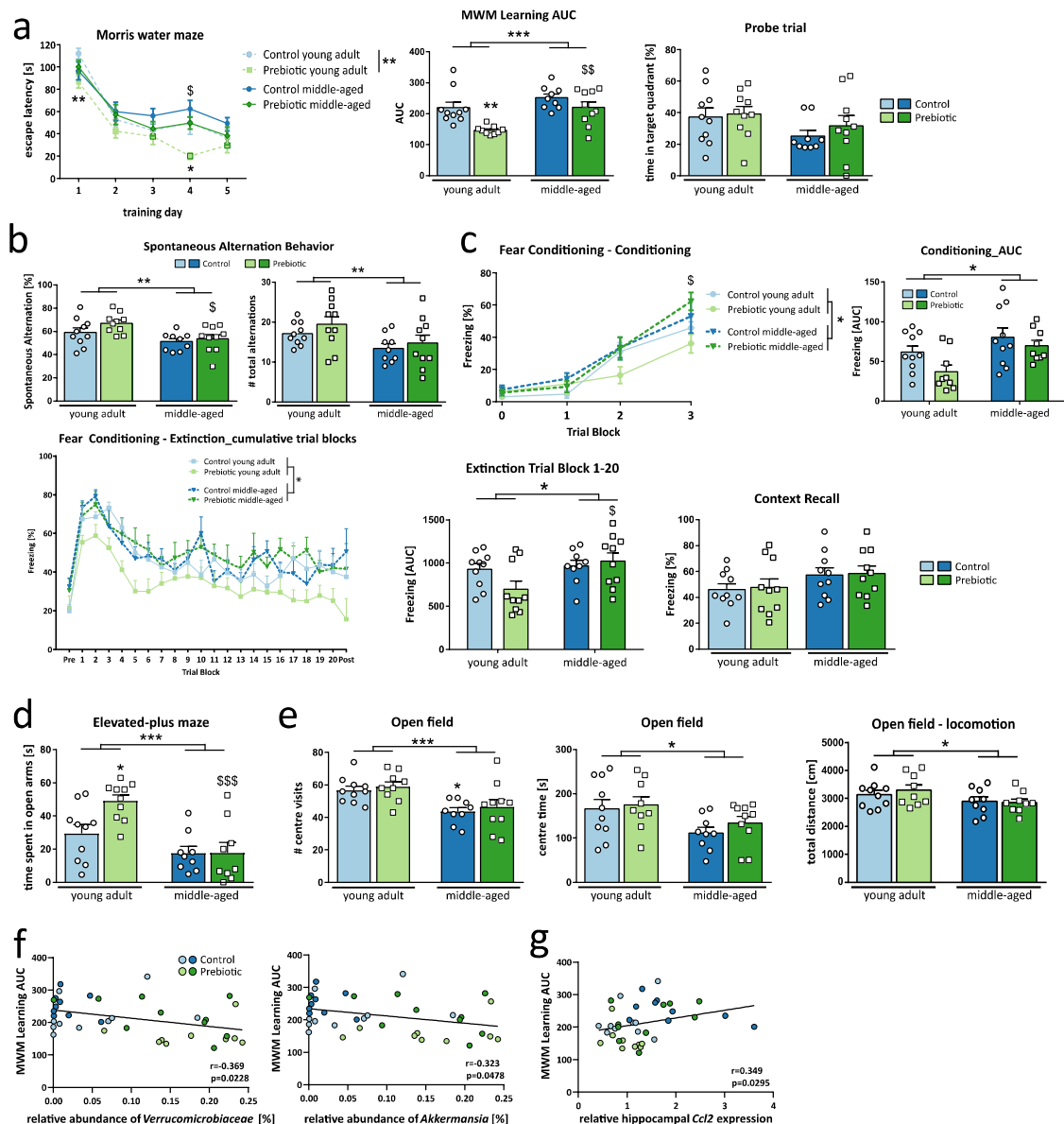


Figure 2.1-3. Prebiotic supplementation improved learning and reduced anxiety-like behaviour in young adult mice. (a) Learning and memory in Morris water maze (MWM). Latency-to-find platform over five training days. Summarized as area-under-the-curve (AUC), as well as the probe trial 24h after the last training day is depicted. **(b)** Short-term memory assessed by Spontaneous Alternation Behaviour (Y-Maze). **(c)** Fear Conditioning: Conditioning (Acquisition, day 1) including AUC. Extinction (day 2) – two consecutive cue presentations were depicted as one trial block. AUC for trial block 1-20 is depicted. Context recall (day 3). **(d)** Time spent in open arms in elevated-plus maze. **(e)** Behaviour in open field. **(f)** Spearman correlation analysis of learning efficacy in Morris water maze (AUC) vs. relative abundance of bacteria from the Verrucomicrobiaceae family and Akkermansia genus **(g)** Spearman correlation learning in Morris water maze vs. hippocampal Ccl2 expression. Mean \pm SEM. n = 9-10. vs. control young adult * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control middle-aged # $p < 0.05$, prebiotic middle-aged vs prebiotic adult: \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$.

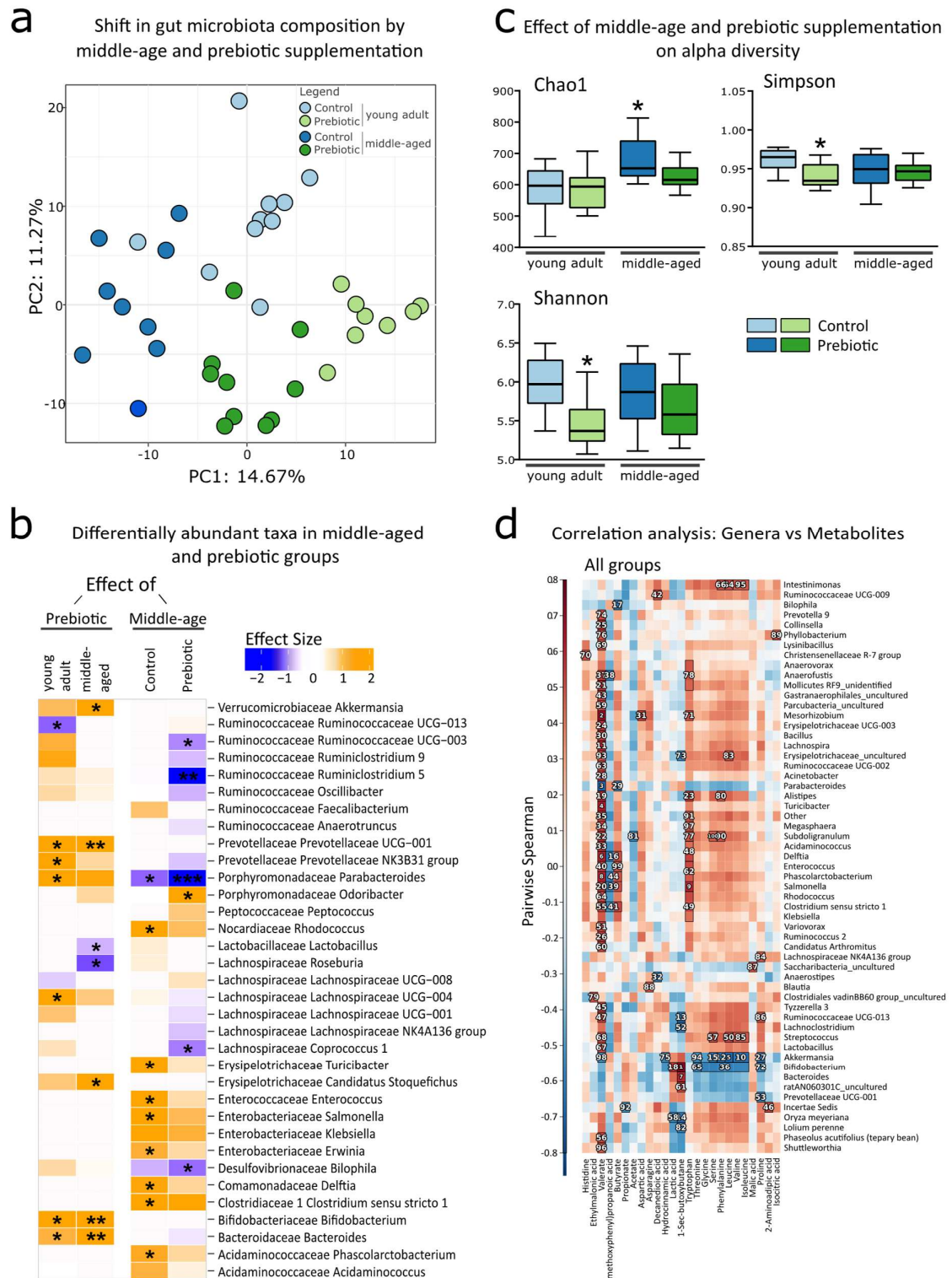


Figure 2.1-4. Middle age and prebiotic treatment have distinct effects on the gut microbiota composition accompanied with changes in short-chain fatty acid (SCFA) profile. (a) PCA plot. (b) Alpha-diversity Indices (Chao1, Simpson, Shannon). (c) Heat

map representing differentially abundant taxa (genus with higher hierarchy family name). Microbes are sorted taxonomically; therefore, genetically closer microbes are closer together. Heat map represents all taxa which reach a Benjamini-Hochberg FDR q value < 0.2 at least once. Asterisks in the heat map represent the following q values: * < 0.1 , ** < 0.01 , *** < 0.001 . (d) Hierarchical All-against-All significance testing (HALLA) representing the 100 strongest significant correlations ($q < 0.2$) between gut microbiota composition and faecal metabolomics. Numbers (1-100) indicate the strongest correlations in a descendant order.

2.1.4.5 Effect of age and prebiotic supplementation on gut microbiota composition and short-chain fatty acid profile in the gut

Principal Component Analysis (PCA) analysis identified structural differences in microbiota across all four groups (PERMANOVA, $p < 0.001$; Figure 2.1-4a). The composition of caecal microbiota was significantly affected by age and by prebiotic supplementation (all $p < 0.05$, pairwise PERMANOVA). Interestingly, no interaction between age and prebiotic was observed, i.e. marked differences between middle-aged and young mice were evident in both control and prebiotic-treated groups, as well as prebiotic supplementation effectively shifted microbiota composition in both young adult and middle-aged animals.

When we looked at structural properties of microbial communities at the genus level, we observed multiple changes in the relative abundance of individual bacterial taxa (Figure 2.1-4b). In particular, middle-aged mice displayed an increase in *Clostridium sensu stricto 1*, *Delftia*, *Salmonella*, *Enterococcus*, *Turibacter* ($q < 0.1$). In contrast, *Parabacteroides* ($q < 0.01$) was decreased in middle-aged control mice. Interestingly, prebiotic supplementation not only increased the abundance of *Bifidobacterium* in young adult but also middle-aged mice ($q < 0.1$ and $q < 0.01$, respectively). In contrast, *Akkermansia* was only increased in middle-aged prebiotic-treated mice ($q < 0.1$). Moreover, prebiotic supplementation increased the abundance of *Prevotellaceae UCG-001* and *Bacteroides* not only in young adult mice but even more pronounced in middle-aged mice ($q < 0.01$, respectively), while *Lactobacillus* and *Roseburia* were decreased in prebiotic-treated middle-aged mice ($q < 0.1$).

The Chao1 index was increased in middle-aged compared to young adult control mice, indicating an increase in overall richness of bacterial species associated with age ($p=0.028$; Kruskal-Wallis post-hoc Dunn's; Figure 2.1-4c). However, the Shannon and the Simpson indices, which take into account the evenness of species abundance, were not affected by age but were reduced following prebiotic supplementation in young adult mice ($p=0.010$ and $p=0.016$, respectively). This suggests that prebiotic supplementation favoured the selective expansion of certain bacterial taxa in young adult animals only.

To identify if changes in gut microbiota composition correlated with faecal metabolomics, we utilized Hierarchical All-against-All significance testing (HALLA). Among others, HALLA identified a negative association between the relative abundance of *Akkermansia*, which was significantly over-represented in prebiotic-treated middle-aged mice, and several amino acids including leucine ($\rho=-0.63$, $p<0.001$, FDR corrected, Figure 2.1-4d), valine and isoleucine ($\rho=-0.60$, $p<0.001$, respectively). Similarly, between *Bifidobacterium*, which was significantly over-represented in prebiotic-treated young and middle-aged mice, and the respective amino acids ($\rho=-0.55$, $p=0.001$).

Prebiotic supplementation increased caecum weight ($F_{(1, 35)} = 88.95$, $p<0.001$; Supplementary Figure S6b) in both young adult and middle-aged mice. Among short-chain fatty acids (SCFAs), caecal butyrate, propionate and valerate levels were affected by either age or prebiotic supplementation. No effect was found on acetate and total-SCFA levels (data not shown). Middle-aged mice exhibited higher butyrate levels than young mice ($F_{(1, 35)} = 16.74$, $p<0.001$; Figure S6c). Prebiotic supplementation increased propionate independent of age ($F_{(1, 35)} = 8.75$, $p<0.001$), with a more pronounced increase seen in middle-aged mice ($p=0.035$). While valerate was increased in middle-aged compared to young controls ($p<0.001$), prebiotic supplementation reduced valerate in both, young adult ($p=0.021$) and middle-aged mice ($p<0.001$).

2.1.5 Discussion

There is a growing appreciation of the role of the gut microbiota in regulating neuroinflammatory responses. The middle-aged brain remains completely understudied regarding this interrelationship. Our data show that middle age is already associated with pervasive alterations in systemic and brain immunity. Targeting the gut microbiome by prebiotic intervention (FOS-Inulin) reversed many of these age-associated neuroinflammatory impairments.

To our knowledge, this is the first study demonstrating the presence of a strong basal and stress-induced (neuro-) inflammatory profile in middle-aged mice (11 months old), although an exaggerated inflammatory response has been previously reported in middle-aged rodents following immune stimulation (Bardou et al., 2013; Lee et al., 2013; Nikodemova et al., 2016). Moreover, our study implicates the gut microbiome in such processes as dietary targeting with prebiotic supplementation counteracted stress-induced peripheral immune cell activation. Following acute stress, we investigated a subtype of neutrophils that express MHC-II, which plays a role in priming of T-cells and therefore provides a link between the innate and the adaptive immune system (Culshaw et al., 2008; Vono et al., 2017). Further research is warranted on the functional characterization of these neutrophils and their impact on the brain in ageing particularly following acute stress.

The gut microbiome has emerged as being essential for brain health in ageing and as a key player in the bidirectional communication across the gut–brain axis (Leung and Thuret, 2015; Dinan and Cryan, 2017). Previous research points out a role of aged microbiota in driving systemic immunity (Fransen et al., 2017; Thevaranjan et al., 2017). In addition, key metabolites which are produced by the gut microbiota following i.e. a prebiotic-enriched diet such as short-chain fatty acids (SCFAs) has been implicated in alleviating stress-induced alteration (van de Wouw et al., 2018). In addition, we show that prebiotic supplementation is capable of dampening age-associated systemic inflammation, particularly $\text{TNF}\alpha$, following stimulation with

Concanavalin A. As ConA stimulates both, T- and NK-cells, it seems that both cell types are in particular sensible to prebiotic treatment in middle-aged mice compared to LPS stimulation which unspecifically stimulates immune cells. We previously showed that prebiotic treatment rescues immune alteration induced by chronic psychosocial stress following ConA stimulation exclusively (Burokas et al., 2017) suggesting that prebiotics might have specific effects on immune priming on T- and NK-cells systemically, and may influence brain function and behaviour as recent research showed a role of T-cell activation in regulating behaviour, anxiety-like and fear-related behaviour (Miyajima et al., 2017), cognition (Derecki et al., 2010) and sociability (Filiano et al., 2016). A critical factor for T-cell activation is the availability of specific amino acids such as leucine (Sinclair et al., 2013). By using HAAIA, we identified strong correlations between prebiotic-driven changes in gut microbiota, *Bifidobacterium* and *Akkermansia* with several amino acids in faecal water, including valine, leucine and isoleucine amongst others. In fact the gut microbiome has been implicated in regulating amino acid availability (Lin et al., 2017). Interestingly, a recent study in a Chinese cohort of middle-aged to elderly individuals found a correlation between *Akkermansia* and CD8+ as well as CD4+ T cells (Shen et al., 2018).

A bidirectional relationship between the brain and the peripheral immune system exists (Varvel et al., 2016), which can promote neuroinflammation and exacerbate neuronal damage in the hippocampus. Recent studies suggest a constant influx of immune cells, inflammatory monocytes (Ly-6C^{hi}-monocytes), into the brain even under steady-state conditions (Möhle et al., 2016; Korin et al., 2017; Mrdjen et al., 2018). Previously these cells were thought to only play a role in inflammatory conditions such as following viral infection and associated encephalitis (Getts et al., 2008) or after social defeat stress (Wohleb et al., 2013; Sawicki et al., 2015). However, recent research suggests that trafficking of Ly-6C^{hi}-monocytes into the brain is crucial for brain plasticity and influence cognitive behaviour (Möhle et al., 2016). This link was mediated by the gut microbiome as antibiotic depletion and subsequent reconstitution of the gut microbiome restored the antibiotic-induced deficits in brain plasticity and cognitive behaviour (Möhle et al., 2016). To

characterize if these Ly-6C^{hi}-monocytes also affect the brain in middle-aged mice before animals were tested behaviourally, we assessed their neuroimmune status in cohort two. Here we show that middle-aged mice exhibited an increased influx of inflammatory monocytes into the brain. To correlate the changes in behaviour with specific neuroimmune markers which link monocyte trafficking to microglia activation, we subsequently analysed targets in the brain at the end of the study. Ly-6C^{hi}-monocytes are hereby recruited to the brain in a CCL2-dependent manner (Mildner et al., 2007; Getts et al., 2008; Sawicki et al., 2015). We show that *Ccl2* is specifically upregulated in the hippocampus of middle-aged mice, but not present following prebiotic supplementation suggesting that this is may be a potential pathway in which gut-microbiota-immune-brain communication can affect brain function and behavioural traits in this key region for learning and memory. However, despite these changes in neuro-immunity, we have not identified any overt cognitive impairments in middle-aged control mice. Although it is worth noting that the dynamics of hippocampal *Ccl2* expression correlated with cognitive behaviour assessed in the Morris water maze paradigm. Interestingly, prebiotic-driven changes in the neuroinflammatory profile are not universal across brain regions as there were no changes in the prefrontal cortex. This is in line with previous findings that there are marked regional differences in microglia activation across brain regions (Grabert et al., 2016). Interestingly, we found that middle-aged mice exhibited increased microglia activation under basal conditions before animals were behaviourally assessed. This subset of inflammatory activated microglia expressed Ly-6C (Mildner et al., 2007; Stirling et al., 2014; Mrdjen et al., 2018) and have been suggested to arise from Ly-6C^{hi}-monocytes (Getts et al., 2008). Recent work has demonstrated a modulatory effect of the gut microbiota on microglia function (Erny et al., 2015; Rea et al., 2016; Vuong et al., 2017). Of note, germ-free mice exhibited deficits in microglia maturation and function while addition of SCFAs rescued these deficits. However, the short-chain fatty acid receptor FFAR2 is actually not present on microglia (Erny et al., 2015), but on monocytes (Ang et al., 2016). Future studies are needed to investigate the mechanistic relationship between these receptors and prebiotic-induced effects on microglia activation across the lifespan.

Microglia activation has been shown to alter cognitive and anxiety-like behaviour (Wohleb et al., 2014; Tay et al., 2017). Here, we show that prebiotic supplementation improves anxiety-like behaviour and cognition in young adult mice. This is in accordance with previous studies which targeted the gut microbiome by dietary interventions in rodents (Savignac et al., 2015; Tarr et al., 2015; Vazquez et al., 2015; Burokas et al., 2017; Mika et al., 2018). Interestingly, studies using a probiotic mix (VSL#3) failed to show improvements in anxiety-related behaviour (Beilharz et al., 2017) suggesting that strain selection is very important and that prebiotics might be a better approach to improve behaviour. Moreover, we show that middle-aged control mice showed a decreased number of centre visits in the open field suggesting increased anxiety-like behaviour (Ennaceur et al., 2008), which may have influenced cognitive performance (Shoji et al., 2016). Middle-aged mice displayed mild cognitive impairments, which were not present following prebiotic supplementation. It is worth noting that neuroinflammation at this stage was not significant enough to manifest in major cognitive impairments. However, our data imply that prebiotic intervention may have some potential to counteract cognitive decline. As the impact of prebiotic supplementation on behaviour, particularly the cognitive tests, is clearly stronger in adult subjects, the data suggests that prebiotics may be less effective as we age. On the other side, a much longer exposure to prebiotics might be needed to achieve significant effects suggesting that supplementation may have to start earlier to be effectively preventative before alterations in the brain occur. This is particularly evident for the behaviour. On the other side, particularly in light of the stress-induced peripheral immune data, the system may need to be challenged to potentially exert negative behavioural effects (Fonken et al., 2018) before prebiotic supplementation can act beneficially (Burokas et al., 2017). Future studies focused on long-term effects of this mid-life microbiota manipulation are now warranted.

We hypothesized that the dysregulated gut-microbiome-brain axis in middle-aged mice can be ameliorated by targeting the gut microbiome with prebiotics known to promote beneficial bacteria like *Bifidobacteria*. It was previously shown that the

prebiotic, inulin, can alter the microbiome composition under pathophysiological conditions such as following high-fat diet (Zhang et al., 2017) or in extreme ageing (Matt et al., 2018); however, its effects remained unexplored in healthy ageing/middle age. In fact, by utilizing FOS-Inulin, we show a profound yet differential alteration of the gut microbiota composition in both young adult but also in middle-aged mice. This was concomitant with a change in short-chain fatty acids with propionate increased in prebiotic-treated middle-aged mice while prebiotic supplementation decreased valerate in both, young adult and middle-aged mice.

Previous research has shown that diet-driven modulation of the gut microbiota by administration of prebiotics can modulate peripheral immune response in the serum of naïve mice (Burokas et al., 2017) and we recently showed that SCFAs attenuate the effect of chronic stress (van de Wouw et al., 2018). It was shown previously that propionate can inhibit the production of pro-inflammatory cytokines (Vinolo et al., 2011). Moreover, *in-vitro* experiments suggests pro-inflammatory capabilities of valerate while it enhanced LPS-induced inflammatory response in a murine N9 microglial cell line (Huuskonen et al., 2004). Although the effects on SCFA levels is relatively modest it is possible that some of the anti-inflammatory effects of prebiotic supplementation might have been mediated by the changes observed in SCFA concentrations.

We have previously reported a shift in microbial composition by prebiotics in adult mice (Burokas et al., 2017) but the impact on middle-aged remained unexplored. Interestingly, we found an increase in species richness in middle-aged mice, which is in line with previous findings in rodents (Scott et al., 2017) and humans (Odamaki et al., 2016). In fact, it has been shown in humans that the gut microbiota remarkably changes with ageing not only in diversity but also representation of specific taxa (Claesson et al., 2011; Claesson et al., 2012; Yatsunenko et al., 2012).

Prebiotic supplementation increased the relative abundance of *Bifidobacterium*, which is in accordance with previous studies in humans (Gibson et al., 1995). Interestingly, *Bifidobacteria* has been reported to be reduced in the elderly (Hopkins et al., 2001). In addition, supplementation increased the relative abundance of *Akkermansia* in middle-aged mice suggesting that prebiotics might promote a young microbiota phenotype, compared to a previous study where *Akkermansia* abundance strongly declined in 12- vs. 4-months-old control mice (van der Lugt et al., 2018). When transferring faecal matter from old mice to young germ-free (GF) mice, *Akkermansia* was lower abundant in those recipients than in GF mice that received young microbiota (Fransen et al., 2017). Interestingly, *Akkermansia* has been associated with immune modulation (Fransen et al., 2017), has shown to protect against inflammation and promote gut health in diet-induced obesity (Everard et al., 2013), and restored intestinal permeability and subsequent immunomodulation in aged mice (Bodogai et al., 2018). Moreover, *Akkermansia* has been found to be enriched in super-centenarians (Biagi et al., 2016). Together with *Bifidobacterium*, *Akkermansia* are claimed as longevity-adapted and possibly health-promoting taxa and therefore might be involved in healthy ageing (Biagi et al., 2016). It is worth noting that learning performance strongly correlated with the abundance of *Akkermansia* suggesting a link between microbiota and cognitive performance. Future studies are warranted to investigate the potential beneficial impact of *Akkermansia* on cognitive performance and healthy ageing.

In conclusion, the present study identified a strong neuroimmune phenotype in middle-aged mice. Moreover, prebiotic-driven changes in gut microbiota composition are beneficial for host health and associated well-being in middle-aged mice (Figure 5). Prebiotic supplementation is capable of altering age-induced changes in brain homeostasis, particularly alleviation of microglia activation, suggesting a preventative strategy towards preservation of cognitive health in ageing. Taken together, the modulatory effects of prebiotic supplementation on monocyte infiltration into the brain and accompanied regulation of age-related microglia activation highlight a potential pathway by which prebiotics can modulate

peripheral immune response and alter neuroinflammation in ageing. Our data thus suggest a novel strategy for the amelioration of age-related neuroinflammatory pathologies and brain function.

2.2 Preventing adolescent stress-induced cognitive and microbiome changes by diet

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2.2.1 Abstract

Psychological stress during adolescence may cause enduring cognitive deficits and anxiety, both in humans and animals that are accompanied by a rearrangement of a multitude of brain structures and functions. A healthy diet is essential for proper brain development and for the maintenance of optimal cognitive functions during adulthood. Furthermore, nutritional components profoundly affect the intestinal community of microbes that may impact on gut to brain communication. We adopted a relatively mild stress protocol, the social instability stress that when repeatedly administered to juvenile rats, modifies cognitive behaviors and plasticity markers in the brain. We then tested the preventive effect of a prolonged diet enriched with the ω -3 polyunsaturated fatty acids eicosapentaenoic, docosahexaenoic, docosapentaenoic acid, and vitamin A. Our study highlighted the beneficial effect of the enriched diet on cognitive memory impairment induced by social instability stress, as rats fed the enriched diet exhibited performance in both emotional and reference memory test indistinguishable from non-stressed rats. Furthermore, the decline of brain derived neurotrophic factor (BDNF) expression in the hippocampus and shifts in microbiota composition of stressed rats were normalized by the enriched diet. The detrimental behavioral and neurochemical effects of adolescent stress as well as the protective effect of the enriched diet were maintained through adulthood, long after the exposure to the stressful environment was terminated. Taken together, our results strongly suggest a beneficial role of nutritional components to ameliorate stress-related behaviors and associated neurochemical and microbiota changes, opening new venues in the field of nutritional neuropsychopharmacology.

2.2.2 Introduction

In rodents, as in humans, adolescence is a time of developmental changes and reorganization in the brain and stress systems, marked by cognitive maturation and behavioral changes (Spear, 2000). Interactions with age-matched conspecifics during adolescence are important for appropriate rodent neurodevelopment and any

alteration to such adolescent social experiences can result in neurobehavioral measurements relevant to anxiety, depression, and substance of abuse (Burke et al., 2017). Preclinical research has focused on earlier and later periods of development, as several reports have demonstrated that early-life stress in rodents as well as in humans represents a neurodevelopmental risk with implications to subsequent cognitive abilities during adulthood (Kaplan et al., 2001; Oomen et al., 2011). Given the paucity of data on the key factors that contribute to the detrimental effects of adolescence stress, no effective strategies have been developed to prevent or cure these problems. In this respect, nutrition is one of the key lifestyle factors that contributes to mental health, and has far-reaching consequences on cognitive functions that extend late in life (Laus et al., 2011; Prado and Dewey, 2014). Among the nutritional components associated with optimal brain functioning, the ω -3 polyunsaturated fatty acids (ω -3 PUFAs) play a critical role in the development and function of the central nervous system (CNS). In animal studies, prenatal deficiency of brain ω -3 PUFAs is associated with enduring neuroanatomical and neurotransmitters alterations, neurocognitive deficits, elevated behavioral indices of anxiety, aggression, and depression (reviewed in (McNamara and Carlson, 2006)) and increased vulnerability to the effect of inflammatory events (Delpech et al., 2015; Labrousse et al., 2018). Recent studies have proven the long lasting, beneficial cognitive effects of a diet supplemented with the ω -3 PUFAs eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) on early stressful events such as maternal separation (Mathieu et al., 2011; Pusceddu et al., 2015), or following ω -3 PUFAs deficiency during the perinatal period (Lozada et al., 2017; Robertson et al., 2017). Also, supplementation with ω -3 PUFAs prevented the emotional and neuronal impairments induced by chronic social defeat in adult mice (Larrieu et al., 2014) and ameliorated memory performance in aged animals (Denis et al., 2013). Another essential nutrient is vitamin A, that through its active metabolite retinoic acid, plays a key role in cognitive functions in adult (Olson and Mello, 2010) and aged rats (Bonhomme et al., 2014). Vitamin A deficiency increased hypothalamus-pituitary-adrenal axis activity and vitamin A-deficient rats showed a delayed and heightened corticosterone response to restraint stress (Marissal-Arvy et al., 2013). On the

contrary, vitamin A supplementation to adult rats prevented age-associated spatial memory decline (Touyarot et al., 2013). A vast literature indicates that ω -3 PUFAs and retinoic acid signaling pathways may act together to modulate memory and synaptic plasticity and indeed, a combined diet enriched with EPA-DHA and Vitamin A had synergistic behavioral and neurochemical effects in middle-aged rats (Letondor et al., 2016). However, the long-lasting effects of a prolonged diet enriched with these micronutrients on the detrimental consequences of adolescence stress is a fairly unexplored field.

The gut microbiota has emerged as one of the key regulators of brain health across the lifespan including adolescence (McVey Neufeld et al., 2016; McVey Neufeld et al., 2016; Dinan and Cryan, 2017). Stress and other insults can seriously impact the composition of the microbiome (Foster et al., 2017) and dietary interventions have been shown to normalize such effects (Pusceddu et al., 2015). Moreover, ω -3 PUFAs deficiency or supplementation have been shown to differentially modulate microbiome composition (Pusceddu et al., 2015; Robertson et al., 2017; Robertson et al., 2017).

In the current study, we hypothesized that a diet enriched in ω -3 PUFAs and Vitamin A may prevent immediate and long-lasting behavioral deficit, neurochemical and intestinal microbiota changes induced by stress during adolescence. We used the social instability stress, a well validated animal model of social stress that produces long-lasting effects on cognitive and emotional responses that may persist for the entire life (McCormick et al., 2015; Burke et al., 2017). We then adopted a multilevel approach at two different ages (immediately after completion of the social instability stress procedure and at adulthood) using a battery of behavioral tests comprehensive of several domains potentially affected by chronic stress: cognition pertaining to emotional and recognition memory, anxiety-like and anhedonia-like responses. We also measured the expression of Brain Derived Neurotrophic Factor (BDNF) that promotes neuronal survival, regulates nerve cells differentiation and may influence cognitive impairment (Buchman et al., 2016), and of synaptophysin, an abundant synaptic vesicle-associated protein involved in synaptic formation (Tarsa and Goda, 2002) in the hippocampus and frontal cortex of adult and

adolescent rats. These brain regions are particularly vulnerable to the negative impact of stress, especially during early-life (Bennett and Lagopoulos, 2014).

In addition, we investigated the short and long-term impact of social instability stress and the dietetic intervention on caecal microbiota composition and short chain fatty acids (SCFA) production. Our findings revealed that the ω -3 PUFAs/Vitamin A enriched diet prevented adolescent stress-induced cognitive and microbiome changes.

2.2.3 Materials and Methods

2.2.3.1 Social Instability stress.

Male Wistar rats arrived to our animal facility at PND 25 and were randomly assigned to three experimental groups: a group of non-stressed animals fed with the control diet (NSCD), a second group subjected to social instability protocol and fed with the control diet (SCD), and a third group also submitted to stressful manipulation and fed with the enriched diet (SED). The social instability stress involves changing the social housing conditions of adolescent rats according to (McCormick et al., 2015). In brief, on each day from postnatal days (PND) 30 to 45, rats were isolated for 1 h in ventilated, round small plastic containers (10 cm in diameter), akin to restraint. After isolation, rats were housed with a new partner undergoing the same procedure in a new cage. The stress regimen occurred at various times during the light cycle to decrease the predictability of the event. After the last isolation on PND 45, rats were returned to their original cage partner. Non-stressed rats were not disturbed except for regular cage maintenance and to be weighed. The consequences of the social instability stress procedure were assessed during adolescence (PND 46-51) and during adulthood (PND 70-76) using a battery of tests comprehensive of several domains affected by chronic stress: cognition (novel object recognition and contextual fear conditioning), anhedonia-like behavior (sucrose preference), anxiety-like behavior (elevated plus maze). Locomotor activity was measured in an open field arena. One day after the end of the behavioral tests, the brains and caecal content were collected for neurochemical determinations and metagenomics analysis, respectively. Different cohorts of animals were used at the two time points. The

experimental timeline is depicted in Fig. 1A. Details of the behavioral tests, western blot and metagenomics analysis are provided in the Supplementary Information. Previous reports indicate that while adult rats readily habituate to this procedure, adolescents show increased corticosterone release in response to repeated changes of cage partners. Consistently, the long-lasting cognitive and emotional alterations observed in adolescent rats subjected to the social instability procedure were not found in adults rats, suggesting that this model may capture adolescent-specific stress reactivity (Hodges and McCormick, 2015; McCormick et al., 2015).

2.2.3.2 Diets composition.

Diets were matched for macronutrients content and the detailed composition is shown in SI Table S1. To prevent oxidation of PUFAs, diets were maintained in air-sealed bags at 4 °C in the dark. Food was changed and weighted every day.

2.2.4 Results

We adopted the social instability protocol (McCormick et al., 2015) consisting of daily isolation followed by change of cage partners from PNDs 30 to 45 (Fig. 2.2-1A). In rodents adolescence is defined by the time elapsed between weaning at PND 21 and the first signs of puberty that in males coincides with preputial separation occurring around PND 42±3 (Spear, 2000). To assess if social instability stress cause acute and long-lasting deficits and if an ω-3 PUFAs/Vitamin A enriched diet (SI Table S1) could ameliorate stress-induced deficits we utilized a battery of measurements and behavioral tests, that were performed starting on the day after completion of the stress protocol at PND 46 (adolescents) or at PND 70 (adults).

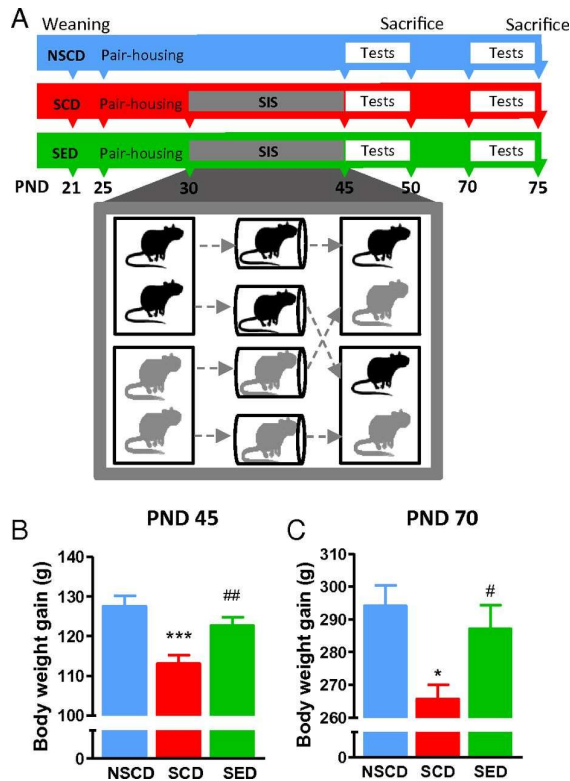


Figure 2.2-1. (A) Time line for the adolescent social instability stress experiment. Adolescent rats were randomly assigned to three experimental groups: NSCD, SCD, and SED. (B and C) Effects of stress and enriched diet on body weight at PND 45 on completion of the stress procedure (B) and at PND 70 (C). $n = 18\text{--}24$ rats/group. *** $P < 0.001$, * $P < 0.05$ vs. NSCD rats; ## $P < 0.01$, # $P < 0.05$ vs. NSCD by one-way ANOVA and the Bonferroni test.

2.2.4.1 Effect of stress and enriched diet on body weight and food consumption

As shown in Fig. 1B, adolescent stressed gained less weight than non-stressed rats ($F_{(2,61)}=9.950$; $P<0.001$), an effect that lasted until adulthood ($F_{(2,59)}=5.262$; $P<0.01$; Fig 2.2-1C) as previously reported (Hodges and McCormick, 2015). This effect was counteracted by ω -3 PUFAs/Vitamin A enriched diet. At both ages, rats ate comparable amounts of food independently of stress and diet (SI Fig. 1S A, B).

2.2.4.2 Enriched diet prevented the cognitive impairments induced by Social Instability Stress.

Two weeks of social instability stress had a negative impact on recognition memory that lasted until adulthood. For short-term memory (1 hr after training), adolescent rats spent significantly more time exploring the novel object regardless of stress or diet (objects $F_{(1,34)}=62.88$, $P<0.001$; condition $F_{(2,34)}=0.0$, $P>0.05$; interaction

$F_{(2,34)}=4.43$, $P>0.05$; Fig. 2.2-2A). Regarding long-term memory (4 hrs after training), Stressed rats fed with Control Diet (SCD) did not discriminate between the two objects (Fig. 2B). The enriched diet fully prevented the stress-induced impairment of object discrimination (objects $F_{(1,30)}=59.11$, $P<0.001$; condition $F_{(2,30)}=0$, $P>0.05$; interaction $F_{(2,30)}=15.01$, $P<0.01$). The cognitive impairment induced by the social instability stress persisted into adulthood and was prevented by the enriched diet administered since adolescence ($F_{(1,30)}=67.45$, $P<0.001$; conditions $F_{(2,30)}=0$, $P>0.05$; interaction $F_{(2,30)}=14.08$, $P<0.001$; Fig. 2.2-2D).

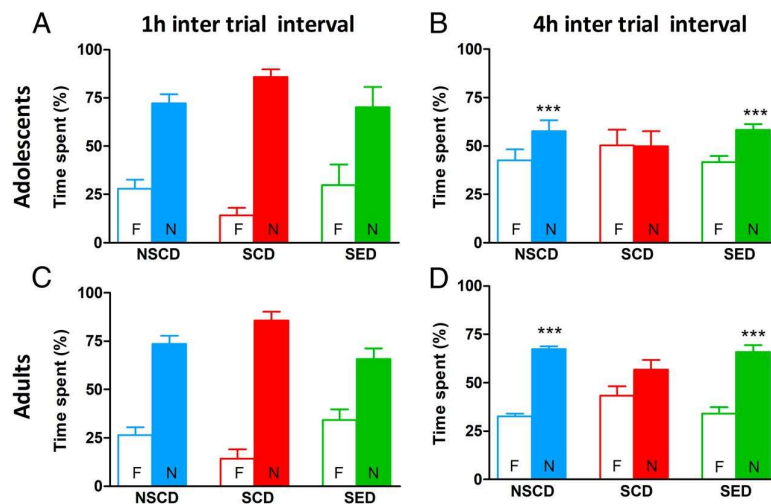


Figure 2.2-2. The enriched diet prevented stress-induced cognitive impairment in the novel object recognition test. (A and C) Stress did not affect the performance of either adolescent or adult rats when the test was performed at 1 h after training. (B and D) Adolescent and adult stressed rats showed memory impairment when tested at 4 h after training, which was prevented by dietary supplementation with ω -3 PUFA/vitamin A. $n = 6-8$ rats/group. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ vs. familiar object within each experimental group by two-way ANOVA and the Bonferroni test.

Furthermore, we tested rats with an emotional arousing training experience that engages both contextual and emotional memory processing: the contextual fear conditioning paradigm. Freezing time obtained during a 3 min re-exposure of rats to the conditioning apparatus 24 hrs after acquisition was used as an index of memory of the aversive experience. As shown in Fig. 2.2-3A, social instability stress did not affect acquisition of fear memory irrespective of diet, whereas SCD adolescent rats

froze less during context retrieval than did Non-Stressed rats fed with Control Diet (NSCD; $F_{(5, 55)}=22.77$, $P<0.001$). The enriched diet restored contextual fear memory expression, as SED rats spent significantly more time freezing at recall than SCD rats which were indistinguishable from NSCD rats. The emotional memory deficit and beneficial effects of the enriched diet were long lasting, as adult Stressed rats fed with Enriched Diet (SED) froze significantly more than SCD rats ($F_{(5, 55)}=47.95$, $P<0.001$; Fig. 2.2-3B)

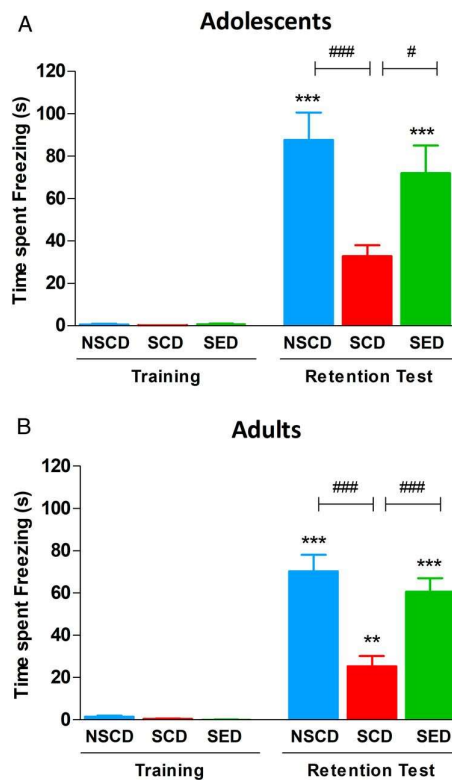


Fig 2.2-3. The enriched diet prevented immediate (A) and long-term (B) stress-induced cognitive impairment in the contextual fear conditioning test. Rat freezing time did not differ at training regardless of treatment condition. When tested at 24 h after training, the SCD rats showed a lower freezing time than the NSCD rats, and the SED rats showed no stress-induced cognitive impairment. $n = 9-10$ rats/group. *** $P < 0.001$, ** $P < 0.01$ vs. respective training; ### $P < 0.001$, # $P < 0.05$ vs. SCD by one-way ANOVA and the Bonferroni test.

2.2.4.3 Enriched diet prevented short and long-term effects of social stress on the expression of BDNF.

To investigate the effect of stress and diet on brain plasticity, we assessed the expression of BDNF in the brain. We found significant differences in BDNF protein levels in the hippocampus of adolescent rats ($F_{(2,27)}=10.33$, $P<0.001$; Fig. 2.2-4A). BDNF expression was decreased in SCD compared to NSCD rats and this effect was prevented by the enriched diet. In the frontal cortex of adolescent rats, stress did not significantly modify BDNF, whereas the enriched diet increased BDNF levels ($F_{(2,27)}=5.808$, $P<0.01$; Fig. 2.2-4B). Interestingly, social instability stress led to long-lasting decrease of hippocampal BDNF while enriched diet prevented the effect of social instability stress ($F_{(2,28)}=6.896$, $P<0.01$; Fig. 2.2-4C). Similar to adolescent rats, BDNF levels were significantly increased following enriched diet in the frontal cortex of adult rats ($F_{(2,24)}=3.680$, $P<0.05$; Fig. 2.2-4D).

In addition, we used Western blot analysis to detect synaptophysin (a glycoprotein associated with presynaptic vesicles) as a marker of synaptic density. Synaptophysin expression of adolescent and adult rats was not significantly affected by stress (SI Fig. S2), in agreement with a previous report (McCormick et al., 2012). In adolescent rats, the enriched diet did not significantly affect synaptophysin expression in the hippocampus ($F_{(2,28)}=2.121$, $P>0.05$), nor in the cortex ($F_{(2,29)}=2.727$, $P>0.05$). In adult rats, though, we observed a significant, diet-induced increase of synaptophysin expression both in the hippocampus ($F_{(2,26)}=8.858$, $P<0.001$) and frontal cortex ($F_{(2,27)}=3.705$, $P<0.05$) which is consistent with the previous observation that long exposure to ω -3 PUFAs increase hippocampal synaptophysin expression (Venna et al., 2009).

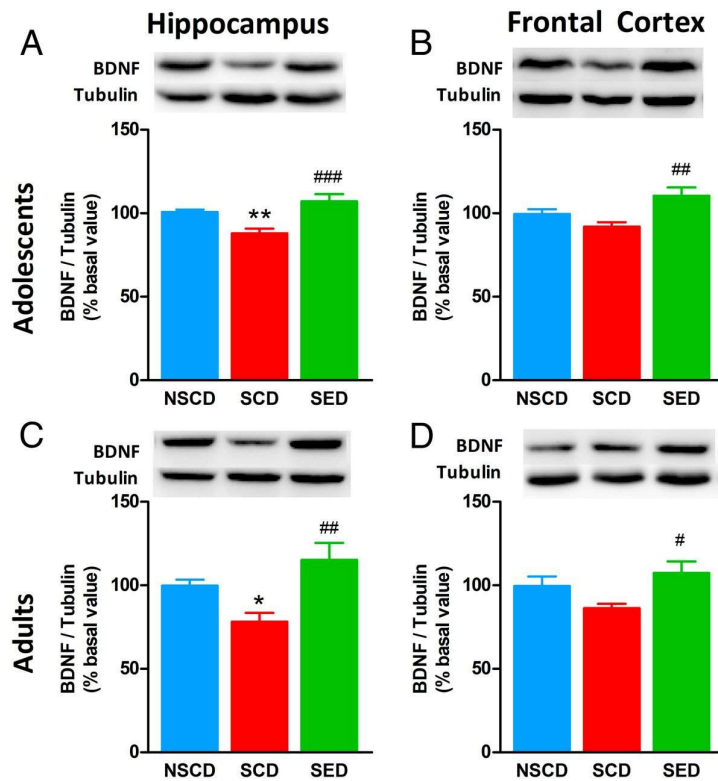


Fig. 2.2-4 The enriched diet restored BDNF expression in the brain of stressed rats. (A and B) Stress decreased BDNF levels in the hippocampus in both adolescent (A) and adult (B) rats. The enriched diet restored BDNF expression to control levels. (C and D) In the prefrontal cortex of stressed rats, the BDNF decrease did not reach statistical significance in either adolescence (C) or adulthood (D); nonetheless, the enriched diet augmented BDNF expression compared with stressed and control rats. (Insets) Representative immunoblots for each experimental group. $n = 8-10$ rats/group. $**P < 0.01$; $*P < 0.05$ vs. NSCD; $###P < 0.001$, $##P < 0.01$, $\#P < 0.05$ vs. SCD by one-way ANOVA and Bonferroni's test.

2.2.4.4 Anhedonia-like behavior.

In agreement with recent data (Marcolin et al., 2018) no differences were observed between stressed and control animals fed with the control diet in terms of preference towards the consumption of a sweetened solution when evaluated at different time points (SI Fig. S3). Neither stress nor the enriched diet had an effect on anhedonia-like behavior, as the preference for a sucrose sweetened drink was not affected by any of the experimental manipulations at any age tested.

2.2.4.5 Locomotor activity and anxiety-related behaviors.

Locomotor activity measured as the distance travelled and time spent moving in an open field was comparable among the groups within each age, although adult rats were less active than adolescents (SI Table S2). Neither stress nor diet affected the number of entries and time spent in the center or periphery of the open field (SI Table S2). Treatment conditions did not affect other exploratory behaviors such as climbing, rearing, grooming indicative of a non-distressed state. Accordingly, there was no effect of treatment conditions on the number of entries and percentage of time spent by adolescent or adult rats in the open arms of the elevated plus maze (SI Table S3).

2.2.4.6 Enriched diet prevented acute stress-induced changes in caecal microbiota composition of adolescent rats.

The enriched diet increased alpha diversity (Chao1) of caecal microbiota of adolescent SED rats compared to both SCD ($P < 0.05$; SI Fig. S4B) and NSCD rats ($P < 0.01$). Consequently, significant differences were found in observed species ($P < 0.05$ and $P < 0.01$, respectively) indicating that the dietary intervention acutely increased microbiota diversity in adolescence irrespective of stress exposure. This was restricted to adolescence as there was no long-lasting effect of diet on diversity into adulthood. Principle Component Analysis (PCA) identified structural differences (beta-diversity) between adolescent NSCD and SCD rats (pairwise PERMANOVA, $P < 0.05$; Fig. 2.2-5A) indicating a shift of the microbiome composition following social instability stress that was almost entirely prevented when stressed rats were fed the ω -3 PUFAs/Vitamin A enriched diet (pairwise PERMANOVA, $P < 0.1$; Fig. 2.2-5A). No long-lasting impact of stress on the composition of the gut microbiota was identified in adult rats (Fig 2.2-5B). However, stressed rats exposed to an ω -3 PUFAs/Vitamin A enriched diet displayed a long-lasting shift in the gut microbiota composition (pairwise PERMANOVA, $P < 0.05$; Fig. 2.2-5B). Compared to adolescent rats, adult rats exhibit a shift in the gut microbiota composition in all three treatment groups (pairwise PERMANOVA, $P < 0.05$; Fig. 2.2-5C-E)

Social instability stress during adolescence significantly modulated a multitude of taxa that were normalized by ω -3 PUFAs/Vitamin A enriched diet (Fig. 2.2-5F). Among

these, SCD rats had a decreased relative abundance of genera of the *Lachnospiraceae* family (in particular *Acetatifactor*, *Anaerostipes* and *Lachnospiraceae* NK4A136 group) and several family members in the *Ruminococcaceae* family (in particular *Ruminococcus* 1), whereas the relative abundance of the *Eubacterium* genus as well as family members from *Coriobacteriaceae* were increased. These changes were largely counterbalanced by the ω -3 PUFAs/Vitamin A enriched diet. Independent of social instability stress, ω -3 PUFAs/Vitamin A enriched diet showed a long-lasting impact only on a few taxa into adulthood (Fig. 2.2-5F).

Social instability stress reduced the concentration of the short-chain fatty acids (SCFAs), butyrate ($P < 0.05$), valerate ($P < 0.05$) and isobutyrate ($P < 0.05$) in adolescent rats (SI Fig. S6). In contrast, SED rats exhibited an increase in isobutyrate ($P < 0.001$) and isovalerate ($P < 0.05$) that led to an overall increase in branched-chain fatty acids (BCFAs; $P < 0.001$). In adult rats, the enriched diet decreased butyrate compared to stressed controls (SI Fig. 4C, $P < 0.05$).

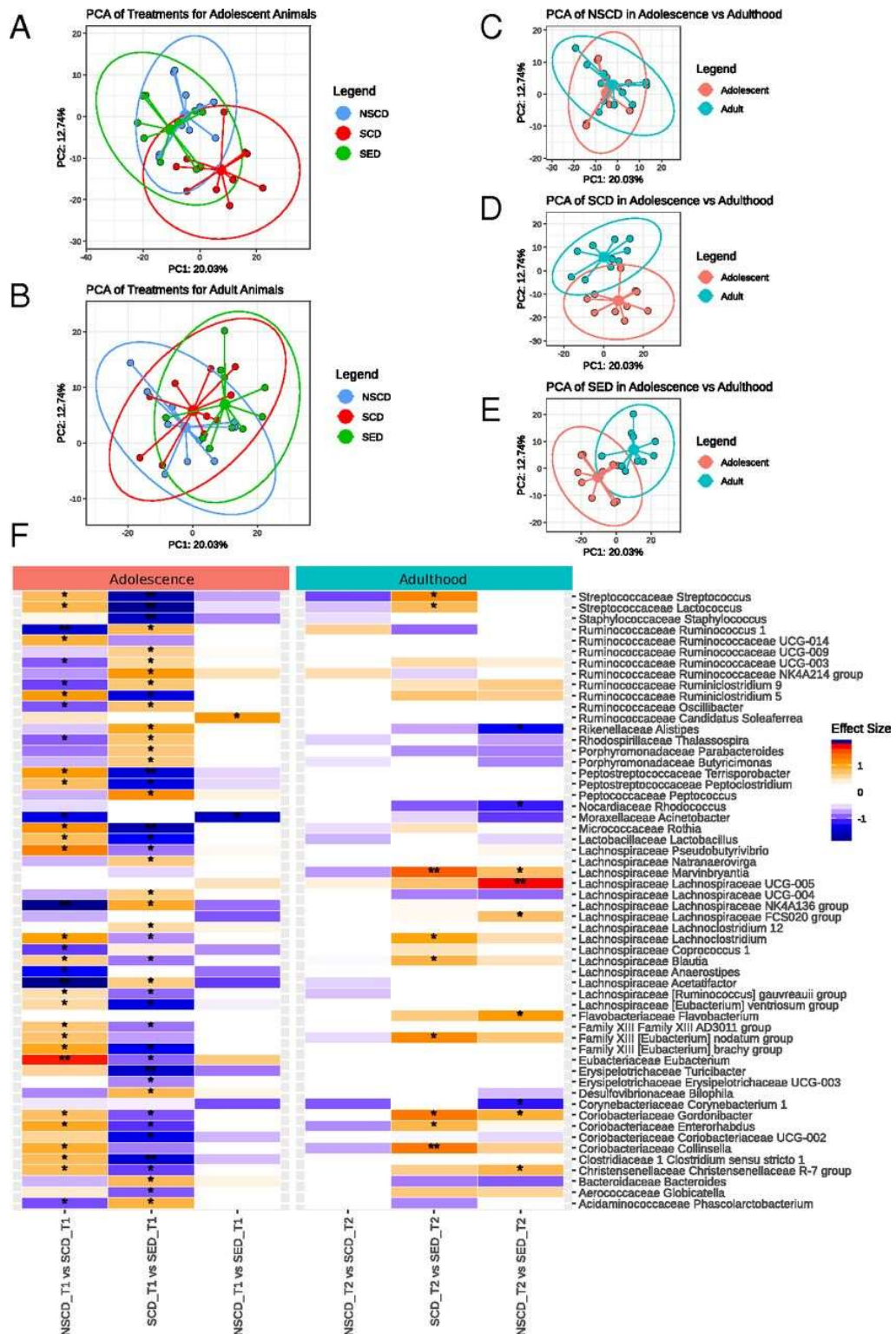


Fig. 2.2-5 Social instability stress shapes the gut microbiome in adolescence. (A and B) PCA plots displaying beta diversity of the gut microbiome in adolescent (A) and adult (B) rats. (C–E) PCA plots comparing beta diversity of the gut microbiome for each treatment between adulthood and adolescence. (F) Changes in gut microbiome composition (genera) in adolescence (T1) and adulthood (T2). Changes in genera in

*each age group are depicted as follows: (i) SCD vs. NSCD, (ii) SED vs. SCD, (iii) SED vs. NSCD. *P < 0.1, **P < 0.01, post hoc Benjamini–Hochberg test.*

2.2.5 Discussion

Nutrition has a fundamental role in maintaining brain health and behavior at critical time periods, especially adolescence (Hueston et al., 2017). In rodents, appropriate essential micronutrient supplementation protects against cognitive decline associated with early life stress (Naninck et al., 2015). Our study corroborates the notion that dietary intervention affects neurobehavioral development (Robertson et al., 2017) by demonstrating for the first time, to the best of our knowledge, that a diet supplemented with the ω -3 PUFAs and vitamin A prevented the deleterious cognitive decline induced by social instability stress during adolescence, and the amelioration was maintained in adulthood. The rationale for using a combination of ω -3 PUFAs and vitamin A stems from recent findings demonstrating a beneficial, synergistic effect of vitamin A and EPA/DHA on behavioral and neurobiological markers of aged rats (Letondor et al., 2016). Multiple levels of interactions occur between ω -3 PUFAs and retinoid signaling, as retinoic acid, the active metabolite of vitamin A, and DHA may bind to common nuclear receptors (de Urquiza et al., 2000). Furthermore, retinoic acid and ω -3 PUFAs have common intracellular signaling pathways such as AKT and ERK1/2 (Masia et al., 2007; Rao et al., 2007), that are known to be activated in several neuronal functions (Tang and Yasuda, 2017).

We found that social instability stress during adolescence caused emotional and recognition memory impairments that were retained until adulthood. These behavioral changes were closely associated with alterations in BDNF expression in the hippocampus and the frontal cortex. Dietary ω -3 PUFAs/Vitamin A exposure from adolescence to adulthood was sufficient to prevent such alterations, and the beneficial outcomes were maintained through adulthood.

2.2.5.1 Enriched diet prevents memory and brain BDNF decline induced by social stress.

Social instability stress in adolescence exerted long lasting effects on aversive and recognition memory. This is in line with previous reports that describe enduring deficits in contextual fear memory in response to adolescence stress (Morrissey et al., 2011). Indeed, the hippocampus is one of the brain structures crucially involved in regulation of stress responses (Reul and de Kloet, 1985). We found that social instability impaired contextual fear memory, a predominantly hippocampus-dependent form of aversive memory (reviewed in (Kim and Diamond, 2002)) in both adolescent and adult rats.

In the novel object recognition test as well, adolescent stressed rats showed long-lasting memory impairments, an effect not previously observed (McCormick et al., 2012). Infact, in McCormick's study (McCormick et al., 2012), stressed rats had hippocampal-dependent spatial recognition impairment, but no reference memory deficit when tested 4 hs after training.

We found a good correspondence between rats' memory performance and BDNF expression in the hippocampus, as both were significantly decreased in stressed adolescent as well as adult rats and the enriched diet prevented both effects. Our results are in agreement with recent observations that ω -3 PUFAs induce BDNF increase in rat hippocampus (Vines et al., 2012). Unequivocal evidence suggests a key role for BDNF in the initiation of fear memory consolidation. Importantly, BDNF enhances and antibodies against BDNF impair fear memory when administered into the CA1 region of the hippocampus (reviewed in (Izquierdo et al., 2016)). Hence, BDNF expression and activity in the hippocampus is required to ensure successful storage for associative memory persistence over days (Bekinschtein et al., 2007). Our behavioral results are in accordance with these observations, as social instability stress reduced hippocampal BDNF and impaired contextual fear memory.

In this regard, we cannot exclude that in our study BDNF modulation in the hippocampus affects also object memory. Although canonically the novel object recognition task is assumed to be largely independent of the hippocampus, some findings are challenging this theory (Clark et al., 2000; Cohen et al., 2013) pointing to

a temporal specificity for hippocampal involvement in object recognition memory (Hammond et al., 2004; Cohen and Stackman, 2015). Nonetheless, our results do not exclude that stressed-rats' cognitive deficit is a global impairment in learning and memory function, rather than being specific to hippocampal responses.

In the frontal cortex BDNF expression is required for fear memory consolidation and expression (reviewed in (Bekinschtein et al., 2014)). Social instability stress did not affect BDNF level significantly in the cortex, of adolescent and adult rats, though the enriched diet augmented BDNF level, presumably concurring in maintaining long-term memory.

2.2.5.2 Enriched diet does not affect behaviors relevant to anxiety and anhedonia.

Social instability- administered during adolescence modifies several social behaviors, as stressed rats spend less time in social interactions with another male, have reduced sexual performance, longer latency to enter the center of an open arena (Green et al., 2013), all validated measures of anxiety-like behavior. Furthermore, the modified social repertoire is evident in adulthood weeks after the end of the stressful procedure (Burke et al., 2017). In our study, we found no difference between stressed and non-stressed rats in the latency to enter the center of the arena or in the number of entries, nor they showed behavioral differences in the elevated plus maze during adolescence or adulthood. One factor contributing to these discrepancies may be strain differences, which are known to be responsible for the anxiety profile (Ramos et al., 1997). In fact, Wistar rats used in our experiments appeared more resilient to the ones commonly used for anxiety-like tests. Other behavioral signs of stress, such as modified grooming, rearing or climbing (Kruk et al., 1998; Fuzesi et al., 2016) were not affected by stress, nor by diet supplementation. Confirming recent data regarding the consumption of natural rewards (Marcolin et al., 2018), neither adolescent nor adult stressed rats did manifest anhedonia-like behavior in the sucrose preference test.

2.2.5.3 Social instability stress during adolescence dramatically altered the gut microbiome which was reversed by ω -3 PUFAs/Vitamin A enriched diet.

Increasing evidence shows that intestinal microbiota influences behaviors relevant to mood and cognitive functions. One of the ways that are proposed for such effects

is by producing metabolites with central effects, among which SCFAs are the most important (Bercik et al., 2011; Bravo et al., 2011; de Lartigue et al., 2011). ω -3 PUFAs are known to have a positive action on the intestinal microbiota increasing the production of SCFAs (reviewed in (Costantini et al., 2017)). Moreover, perturbations of the microbiota during adolescence have been shown to result in enduring social and cognitive deficits (Desbonnet et al., 2015).

Psychological stress (ranging from restraint stress to maternal separation and overcrowding) has been shown to alter microbiota composition (O'Mahony et al., 2009; Bailey et al., 2011; Hsiao et al., 2013; De Palma et al., 2014; Bharwani et al., 2017). Social instability stress induced striking differences in the gut microbiome composition of adolescent rats which were partially reversed by an ω -3 PUFAs/Vitamin A enriched diet. Adolescence stress resulted in a decreased abundance of several genera within the *Ruminococcaceae* and the *Lachnospiraceae* family. The functional consequences of such changes are not clear at this stage but it is worth noting that both, *Ruminococcaceae* and *Lachnospiraceae*, were found to be decreased in patients with depressive disorders (Jiang et al., 2015). In contrast, the *Eubacterium* genus was increased in stressed rats fed with control diet. *Eubacteriaceae* were found to be increased in rats with experimental colitis and when additionally exposed to stress (Konturek et al.) conditions where *Lachnospiraceae* were decreased (66). *Coriobacteriaceae*, in particular *Enterorhabdus*, were increased in SCD rats. This is in line with studies implicating *Coriobacteriaceae* with colonic health and inflammation (Morgan et al., 2012). Nonetheless, the enriched diet was sufficient to prevent such modifications. However, despite the stress-induced changes and independently of the diet, the intestinal microbiota during adulthood recovered the core microbiota composition characteristic of adult animals (Flemer et al., 2017). Interestingly, no long-lasting impact of stress was identified in adult rats. However, stressed rats exposed to an ω -3 PUFAs/Vitamin A enriched diet displayed a long-lasting shift in the gut microbiota composition. A limitation of our current study is that we cannot definitely prove a causal correlation between changes in microbiota and cognitive performance at this juncture. However, given that the microbiome has been implicated as a conduit for

the positive effects of nutrition on host health, modulation of the microbiota is a plausible mechanism as to how nutritional interventions may reduce the effects of stress (Sandhu et al., 2017), as we found that both diet and stress induced effects on both the microbiome and behavioral/neurochemical measures of cognitive functions. We also found that the enriched diet increased the production of branched SCFA whereas unbranched SCFA were unchanged. It was previously shown that SCFA levels strongly correlated with improvement in tests of anxiety- and depression-like behaviors induced by prebiotic diet (Burokas et al., 2017) and that SCFAs can reverse the enduring effects of social stress in adulthood .

2.2.5.4 Conclusion

Stress, emotional instability, impulsivity are all enhanced during adolescence and inadequate nutrition may exacerbate such conditions. Moreover, there is a growing link between changes in gut microbial composition and brain health in adolescence. Clinical studies have shown that young adults who endured environmental or psychosocial stressors during development or have low blood ω -3 PUFAs levels are often diagnosed with psychiatric disorders or cognitive impairments (Espejo et al., 2007; Heim et al., 2008; Kuratko et al., 2013; Montgomery et al., 2013; Joffre et al., 2014). Our study thus provides the first preclinical evidence that ω -3 PUFAs and vitamin A supplementation is sufficient to prevent long-lasting cognitive disturbances and modulate microbiota composition that accompany repeated, prolonged stressful stimuli during adolescence. Optimization of dietary components that affect brain development suggests the likelihood that we may improve our cognition throughout life.

2.3 Polyphenols selectively reverse early-life stress-induced behavioural, neurochemical and microbiota changes in the rat

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2.3.1 Abstract

There is a growing emphasis on the role of the microbiota-gut-brain axis as modulator of host behaviour and as therapeutic target for neuropsychiatric disorders. In addition, accumulating evidence suggests that early-life stress can exert long-lasting changes on the brain and microbiota, and this early adversity is associated with increased risk for developing depression in later life. The maternal separation (MS) model in rats is a robust paradigm to study the effects of early-life stress on the microbiota-gut-brain axis. Recently, we have shown that polyphenols, naturally occurring compounds associated with several health benefits, have anti-stress effects in *in vitro* models. In this study, we assess the therapeutic potential of a variety of both flavonoid and non-flavonoid polyphenols in reversing the impact of MS on behaviour and the microbiota-gut-brain axis.

Rats underwent a dietary intervention with the naturally-derived polyphenols xanthohumol and quercetin, as well as with a phlorotannin extract for 8 weeks. Treatment with polyphenols prevented the depressive and anxiety-like behaviours induced by MS, where xanthohumol effects were correlated with rescue of BDNF plasma levels. In addition, MS resulted in altered brain levels of 5-hydroxyindoleacetic acid (5-HIAA) and dopamine, accompanied by abnormal elevation of plasma corticosterone. Although polyphenols did not reverse neurotransmitter imbalance, xanthohumol normalised corticosterone levels in MS rats. Finally, we explored the impact of MS and polyphenolic diets on the gut microbiota. We observed profound changes in microbial composition and diversity produced by MS condition and by xanthohumol treatment. Moreover, functional prediction analysis revealed that MS results in altered enrichment of pathways associated with microbiota-brain interactions that are significantly reversed by xanthohumol treatment. These results suggest that naturally-derived polyphenols exert antidepressant-like effects in MS rats, which mechanisms could be potentially mediated by HPA regulation, BDNF levels rescue and modulation of the microbiota-gut-brain axis.

2.3.2 Introduction

Stress-related psychiatric disorders including depression and anxiety are currently a major public health concern. Indeed, the World Health Organisation (WHO) has predicted that depression will be the second largest cause of disability by 2020 (Johnston et al., 2019). On the other hand, major depressive disorder is thought to result from the complex interplay of multiple inherited genetic factors and subsequent exposure to a wide range of environmental variables throughout life (aan het Rot et al., 2009); therefore, the search for adequate treatments is a great challenge as no established mechanisms have yet been determined (Berton and Nestler, 2006). Based on these observations and considering that depression has an inconsistent response to treatment, the development of new antidepressant strategies is increasingly being considered as a critical focus of research.

It is well known that stressful events in early life can exert long-lasting changes in brain structure and function later on (Cryan and Dinan, 2013) and accumulating evidence indicates that this early life adversity is associated with an increased risk for developing depression (Chapman et al., 2004; Heim and Binder, 2012). For instance, inadequate maternal care has been linked to developmental, emotional and social deficits in humans (Field, 1998). In rodents, the maternal separation (MS) model is a well-described paradigm used to investigate the neurobiological and behavioural consequences of early life stress (O'Mahony et al., 2011; Nishi et al., 2014; Rincel and Darnaudery, 2019). For this reason, the MS model has been used to study various psychiatric conditions, especially depression (Meaney et al., 1996; Vetulani, 2013) (Wieck et al., 2013).

The microbiota-gut-brain axis describes the complex bidirectional communication system that exists between the central nervous system (CNS) and enteric microbiota; involving endocrine, immune and neural pathways (Rhee et al., 2009; Foster et al., 2017; Cryan et al., 2019). Accumulating research has focused on the impact of the microbiota on CNS function and stress perception, and its consequences for behaviour (Cryan and Dinan, 2012). Indeed, top down activation of the CNS can influence gut neuromotor and secretory function, immunity and microbiota

composition during stress (De Palma et al., 2014; Foster et al., 2017). In this regard, early-life stress models such as MS have long-term impact on the gut microbiota, which correlate with increased HPA axis activity and behaviour (Bailey and Coe, 1999; O'Mahony et al., 2009). Moreover, the MS model is sensitive to reversal treatments that target the gut microbiota (Gareau et al., 2007; Fukui et al., 2018; Cowan et al., 2019; McVey Neufeld et al., 2019; O'Mahony et al., 2019).

The emerging and compelling evidence for nutrition as a crucial factor in the high prevalence and incidence of mental disorders suggests that changes in diet are a viable strategy for improving mental health and treatment of psychiatric disorders including anxiety and depression (Jacka et al., 2014; Lai et al., 2014; Spencer et al., 2017; Larrieu and Laye, 2018; Adan et al., 2019; Dinan et al., 2019). For instance, dietary polyphenols are a group of naturally occurring phytochemicals which are present in high amounts in fruits and vegetables and are characterised by the presence of multiple hydroxyl groups on aromatic rings (Vauzour, 2012). Several studies have focused on the potential of polyphenolic compounds in protecting cognitive function and reducing risk for developing neurodegenerative disorders (Spencer, 2008). In particular, some pre-clinical studies have confirmed the antidepressant capacity of polyphenols in different animal models (Anjaneyulu et al., 2003; Kulkarni et al., 2008; Yi et al., 2008). Moreover, dietary polyphenols are capable of modulating the composition of the gut microbial community by inhibiting or stimulating the growth of certain bacteria (Lee et al., 2006). Hence, there is increasing interest in using polyphenols to target the microbiota-gut-brain axis to treat mental disorders (Filosa et al., 2018; Matarazzo et al., 2018).

Polyphenolic compounds are characterised as having different functional activity depending on their chemical structure (Manach et al., 2004; Vauzour et al., 2010). For instance, phlorotannins are a type of polyphenolic tannins found in marine brown algae, which have been shown to possess anti-oxidant activity, as well as beneficial effects for different diseases such as cancer, cardiovascular problems and diabetes (Kim and Himaya, 2011). Other polyphenols can only be isolated from specific sources. Xanthohumol, for example, is described as a prenylated chalcone, a principal component of the female hop plant, *Humulus lupulus* (Stevens and Page, 2004).

Some health benefits associated with xanthohumol intake include anti-inflammatory and neuroprotective effects (Liu et al., 2015). In contrast, some members of the flavonoid family like quercetin are widely distributed in nature (Manach et al., 2004). Quercetin is one of the most studied polyphenols and has been demonstrated to confer protection against certain types of cancer, cardiovascular and neurodegenerative disorders (Boots et al., 2008).

Recently, we showed that across a wide number of polyphenols, xanthohumol and quercetin were able to reverse the impact of corticosterone exposure in primary cortical neurons (Donoso et al., 2019). Moreover, although the antidepressant effects of several polyphenols have been studied in different preclinical studies (Xu et al., 2005; Bhutada et al., 2010; Liu et al., 2014), their therapeutic effects have not yet been examined in models of early life stress, nor the mechanisms underlying the polyphenol-mediated alleviation of mood. Therefore, the purpose of this study was to explore the therapeutic effects of different naturally derived polyphenols, including phlorotannins, xanthohumol and quercetin in the MS model in rats. In addition, the consequences of MS and polyphenol diet intervention on different aspects of the microbiota-gut-brain axis were explored.

In this regard, we evaluated important components involved in the regulation of this axis, including BDNF, which is a crucial neurotrophin associated with plasticity and neuronal survival (Brunoni et al., 2008; Lee and Kim, 2010); the assessment of neurotransmitter concentrations in the brainstem, an important brain locus for monoaminergic transmission and which is implicated in mood disorders (Sasaki et al., 2008), as well as highly influenced by the gut microbiota (Strandwitz, 2018); and the response to acute stress through the determination of plasma corticosterone, the main rodent stress hormone (de Kloet et al., 2005; Joels et al., 2018). Finally, we evaluated the consequences of MS and diet intervention with polyphenols on the gut microbiota abundance and through the determination of short-chain fatty acids (SCFAs), microbial metabolites thought to play a critical role in gut-brain communication (van de Wouw et al., 2018; Dalile et al., 2019). Together, findings from this study have the potential to provide new insights into the potential therapeutic effects of polyphenols and the role of the microbiota-gut-brain axis in

stress-related disorders, and add an important direction to future dietary advice on optimal nutrition for mental health and to counter the enduring impact of early life adversity.

2.3.3 Methods

2.3.3.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork. Pregnant Sprague Dawley dams weighing 250–300 g were bred in-house in the Biological Services Unit facility, University College Cork. The pups were housed with their mothers in plastic cages (15 × 22 × 9 cm) in a temperature and humidity controlled room on a 12-h light, 12-h dark cycle (lights on from 7.00–19.00 h). Food and water were available ad libitum.

2.3.3.2 Drugs

Quercetin (Q4951) was purchased from Sigma. Xanthohumol (A-4-2014) was provided by Hopsteiner, GmbH (Mainburg, Germany). Phlorotannin-rich extract from *Fucus vesiculosus* (Gite et al., 2019) was obtained from National University of Ireland, Galway (Galway, Ireland). All diets were prepared by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany). The resulting grain based chows were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids).

2.3.3.3 Maternal separation procedure

MS was performed as previously described (O'Mahony et al., 2009; Pusceddu et al., 2015). Briefly, pups were separated from their mother as a whole litter and placed into plastic cages maintained at 30 – 33 °C in a separate room to prevent communication through ultrasonic vocalisation (Hofer et al., 1994). Following the 3-hour separation, pups were returned to their original home cage with their mother. This procedure was repeated each day (9.00am–12.00pm) from post-natal day (PND) 2 through PND12. NS-Control rats consisted of non-handled pups, left untouched by the experimenter, and with their respective mothers. After postnatal day 12, pups

were left undisturbed except for routine cage cleaning every two days. At weaning, male rats were group-housed (2 – 4) in large cages.

2.3.3.4 Treatments

The rats were randomly assigned into five different experimental groups [1] NS-Control diet (n = 12); [2] MS-Control diet (n = 12); [3] MS-Phlorotannins 0.03% (n = 10); [4] MS-Xanthohumol 0.015% (n = 10); [5] MS-Quercetin 0.03% (n = 10). Dietary intervention of polyphenols, delivered ad libitum in food, began once the animals were eight weeks old and continued for eight weeks. The concentrations for the polyphenols tested were calculated based on doses previously reported in animal models and considered the average daily food intake and body weight of Sprague Dawley rats aged between 9 and 16 weeks (Laaksonen et al., 2013). Estimated doses are as follows; quercetin 20 mg/kg/day (Haleagrahara et al., 2009); xanthohumol 10 mg/kg/day (Ceremuga et al., 2013); phlorotannins 20 mg/kg/day (Ahn et al., 2017). In the interest of reduction in the 3Rs a number of other interventions were also run contemporaneously with the control and treatment groups used here (Egerton *et al.* unpublished).

2.3.3.5 Elevated plus maze

The elevated plus maze (EPM) is one of the most commonly used rodent tests for assessing anxiety and was performed as previously described (Cryan et al., 2004; Pusceddu et al., 2015). Briefly, the maze consisted of two open arms (51 × 10 cm; 5 lux) and two enclosed arms (51 × 10 × 41 cm) that all extended from a common central platform (10 × 10 cm). The apparatus was elevated 55 cm above the floor on a central pedestal. Animals were habituated to the testing room for 30 min prior experiment. At week 12, animals were placed in the centre of the maze facing an open arm to begin. Animal behaviour was recorded for 5 min. Frequency of open and closed arms entries were scored, as well as percentage time spent in each arm.

2.3.3.6 Open field test

The open field test (OFT) is commonly used as a mechanism to assess anxiolytic effects of compounds (Seibenhener and Wooten, 2015). Briefly, at week 13 rats were placed in the centre of a white open field arena (60 × 40 cm; 60 lux) and observed for

10 min. Animals were habituated to the test room for 30 min prior to the experiment. All trials were conducted between 9.00am and 1.00pm. The arena was cleaned with 70% ethanol to avoid smell cues between each trial. At the end of each trial, animals were returned to their cages. Distance moved, velocity, percentage of time spent in inner zone, and frequency of inner zone entries were analysed and recorded using a tracking system (Ethovision XT 13, Noldus).

2.3.3.7 Forced swim test

The forced swim test (FST) is the most widely used model for predicting antidepressant activity in rodents, and increased immobility in this test is generally considered to reflect a state of behavioural despair (Porsolt et al., 1978). Briefly, at week 15 a modified rat FST protocol (Slattery and Cryan, 2012) was used to determine the antidepressant effects of polyphenols in rats. On day one, rats were placed individually in glass cylinders (H: 45 cm; D: 20 cm) filled with water to a depth of 30 cm at 24 ± 1 °C for a 15 min pre-test period. The rats were removed from the water, dried and placed in their home cage. The cylinders of water were changed between each trial. 24 hours after the pre-test, the rats were again placed in the swim apparatus for 5 min and behaviours were monitored from above with a video camera for subsequent analysis. Behaviours rated include immobility, climbing and swimming (scoring of behaviours was blind to the experimental conditions). The 5-min session was scored using a time-sampling technique, whereby the predominant behaviour in each 5-s period of the 300-s trial was recorded. Climbing behaviour consisted of upward-directed movements of the forepaws along the side of the cylinder. Swimming behaviour was defined as movement (usually horizontal) throughout the cylinder. The rat was considered to be immobile when the only activity observed was that which was required by the rat to keep its nose above water.

2.3.3.8 Plasma corticosterone determination

Blood sample collection was performed as previously described (Pusceddu et al., 2015). Briefly, blood samples were collected on day one of FST via a tail-tip incision at five different time points: immediately before (baseline), 30 min, 60 min, 90 min and 120 min after the test was started. Approximately 200 µl of blood was collected

in a tube containing 10 μ L of EDTA 0.1 M to avoid coagulation. Blood plasma was obtained by centrifugation at $3500 \times g$ at 4 °C for 15 min. Corticosterone levels were measured using the Corticosterone EIA kit (Enzo) according to the manufacturer instructions, and absorbance signal was detected with a conventional plate reader (Synergy HT, Biotek).

2.3.3.9 Plasma BDNF measurement

Immediately after sacrifice, trunk blood was collected in EDTA Vacutainer tubes. Blood plasma was obtained by centrifugation at $3500 \times g$ at 4 °C for 15 min. Protein levels of brain-derived neurotrophic factor (BDNF) were determined using an electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer's protocol. BDNF levels were determined and analysed using the MSD QuickPlex SQ 120 Instrument.

2.3.3.10 Brain monoamines concentration

The monoamine neurotransmitters noradrenaline (NA), serotonin (5-HT), dopamine (DA) and their metabolites 5-HIAA and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in the brainstem using high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Clarke et al., 2012; Pusceddu et al., 2015). Briefly, samples were homogenised in mobile phase (consisting in 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate monohydrate, 5.6 mM 1-octanesulfonic acid, 0.01 mM EDTA, 11.1% (v/v) methanol, and 0.1 μ g/mL of N-Methyl 5-HT as internal standard and adjusted to pH 2.8). Then samples were centrifuged $14000 g$ for 15 min at 4 °C, and 20 μ L of this supernatant was injected onto the HPLC system (consisting in a CBM-20A system controller, a EC3000 Recipe amperometric detector, a LC-20AD XR pump, a CTO-20A column oven at 30 °C, a SIL-20AC XR autosampler, and a Prominence DGU-20A3 degasser). A reverse-phase column (Kinetex 2.6u C18 100A 100 mm X 4.6 mm, Phenomenex) was employed in the separation using a flow rate of 0.9 mL/min. Each neurotransmitter was identified through their characteristic retention times and their concentration was determined using the ratios of peak heights of analyte versus internal standard provided by the LabSolutions software (Shimadzu). Results were expressed as nanograms of neurotransmitter per grams of fresh tissue.

2.3.3.11 Gut microbiota 16S rRNA sequencing

Microbial DNA was isolated from frozen faecal samples using the QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. DNA concentration and quality was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S metagenomic sequencing library protocol, and PCR reactions were performed with the KAPA HiFi HotStart PCR Kit (KAPA Biosystems). PCR products were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter Life Sciences). This was followed by indexing PCR which attached Nextera XT barcodes and Illumina sequencing adapters to the 5' overhangs and another round of AMPure XP clean-up. Finally, samples were sequenced on the MiSeq™ System (Illumina®), using a 2 x 250bp cycle kit, following standard Illumina sequencing protocols.

2.3.3.12 Short chain fatty acid determination

The SCFAs acetate, propionate, butyrate, and valerate, as well as the total branched chain fatty acids (BCFAs) were measured in caecal content using gas chromatography flame ionisation detection (GC-FID) as previously reported (van de Wouw et al., 2018). Briefly, samples were vortexed with Milli-Q water (1:10 w/v), left to stand for 10 min at room temperature, and then centrifuged at 14000 *g* for 5 min. The supernatant was filtered (0.2 µm) before transfer to a GC glass vial, and 2-ethylbutyric acid (Sigma) was added as internal standard. SCFA concentrations were measured using a Varian CP-3800 GC flame-ionization system, fitted with a Zebron ZB-FFAP column (30 m × 0.32 mm × 0.25 µm; Phenomenex) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was used as the carrier gas at a flow rate of 1.3 ml/min. The initial oven temperature was set at 100 °C for 0.5 min, raised to 180 °C at 8 °C/min and held for 1 min, then increased to 200 °C at 20 °C/min, and finally held at 200 °C for 5 min. The temperature of the injector and the detector were set at 240 °C and 250 °C respectively. A standard curve made from a standard mix of acetic acid, propionic acid, n-butyric acid and iso-butyric acid (Sigma) at seven concentrations. Peaks were integrated by using the Varian Star Chromatography

Workstation version 6.0 software. Standards were included in each run to maintain calibration.

2.3.3.13 Statistical analysis

Statistical analysis was performed using the software SPSS 24.0, and the results were presented as mean \pm SEM. MS-control group and NS-control group were compared using independent T-test to assess the MS effect. All MS groups were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. A *p*-value of 0.05 was considered statistically significant. FLASH was used to assemble paired-end reads. Further processing of paired-end reads including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (version 1.9.0). Denoising, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit). OTUs were aligned using PyNAST, and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Statistical microbiome analysis was carried out in R (version 3.6.1) with Rstudio (version 1.2.1335). OTUs unknown on a genus level were excluded, as well as OTUs available in two or fewer samples. The ALDEx2 library (Fernandes et al., 2014) was used to compute the centred log-ratio transformed values of the remaining taxa. For principal component analysis, a pairwise implementation of the `adonis()` PERMANOVA function in the `vegan` library (Oksanen et al., 2017) followed by the Bonferroni-Holm correction was used to test for difference in β -diversity in terms of Aitchison distance. Differential abundance was assessed using a pairwise implementation of the `aldex.test()` function, followed by Benjamini-Hochberg correction. In these cases, a *q*-value < 0.1 was considered significant. α -diversity was computed using the `iNEXT` library (Hsieh et al., 2016).

2.3.3.14 Functional prediction of Gut-Brain modules

The Piphillin webservice (Iwai et al., 2016) was used to infer the functional metagenome per sample in terms of KEGG orthologues. Next, these KEGG orthologues were processed using the `omixer` library in R (Darzi et al., 2016) in order to calculate abundance of gut-brain-modules (GBMs) (Valles-Colomer et al., 2019) and gut-metabolic modules (GMMs) in these samples. Then, the same

implementation from ALDEx2 was used to assess differential abundance. Scripts are publicly available on GitHub: <https://github.com/thomazbastiaanssen/Tjazi> doi: 10.5281/zenodo.1480804

2.3.4 Results

2.3.4.1 Polyphenols reversed MS-induced depressive-like behaviours

To investigate the therapeutic effects of the dietary interventions with polyphenols from MS-induced behavioural despair, animals were subjected to a battery of behavioural tests to examine depressive- and anxiety-like behaviours. Firstly, animals did not differ in terms of body weight across the different experimental groups throughout the duration of the treatment (Fig. 1B and C). In FST, analysis yielded a significant effect of MS compared to NS-control group on the time spent immobile ($t_{22} = -2.349$; $p = .028$) and swimming ($t_{22} = 2.611$; $p = .016$) (Fig. 2.3-2A). MS animals exhibited improved depressive-like behaviours with xanthohumol; moreover, quercetin and phlorotannins significantly decreased immobility time in the FST ($F_{3,36} = 4.425$; $p = .05$ and $p = .002$ respectively). In addition, treatment with phlorotannins increased swimming time compared to the MS-control group ($F_{3,36} = 2.984$; $p = .008$) (Fig. 2.3-2A).

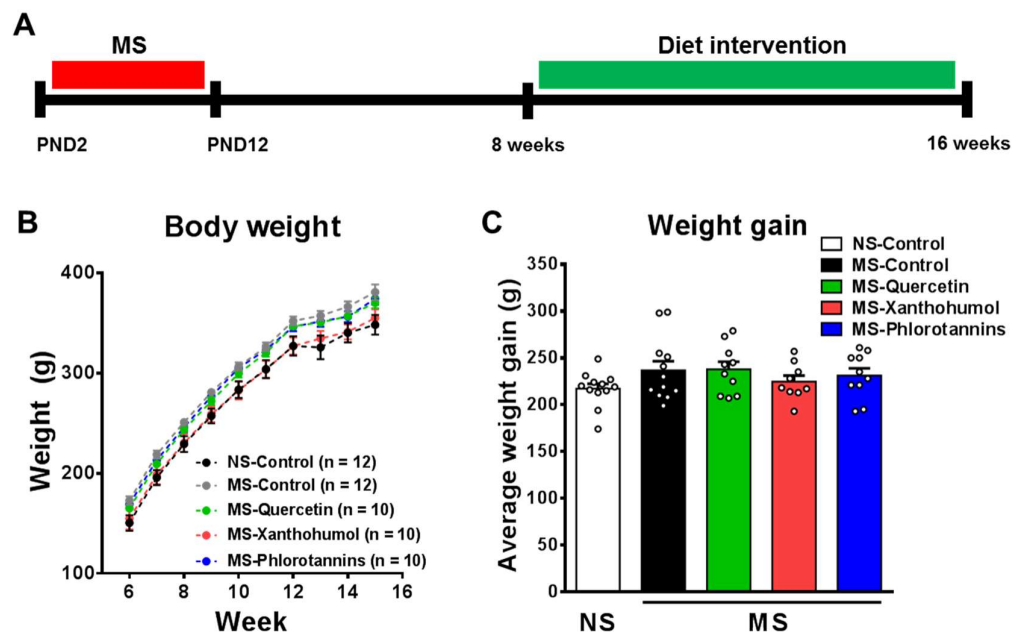


Figure 2.3-1 Diet intervention with polyphenols did not affect body weight. (A) Schematic representing the experimental timeline. **(B)** Body weight was measured weekly from 6-week old until the end of the diet intervention. **(C)** The weight gain was calculated as the difference between the first body weight record (6 weeks) and the last measurement (15 weeks).

2.3.4.2 Polyphenols showed anxiolytic potential in MS animals

MS-induced anxiety-like behaviour in the OFT by significantly reducing the time spent in the centre ($t_{21} = 2.156$; $p = .025$), as well as in the number of entries in the centre of the arena ($t_{21} = 2.855$; $p = .009$) (Fig. 2.3-2D and 2.3-E). Administration of quercetin in MS rats resulted in a significant increase in the number of entries in the open arms compared to the MS-control group ($F_{3,38} = 2.714$; $p = .040$), which was associated with an anxiolytic effect (Fig. 2.3-2G). Interestingly, treatment with phlorotannins ameliorated MS-induced anxiogenic effects in both, time in centre ($F_{3,37} = 2.297$; $p = .025$) and in entries into the centre ($F_{3,37} = 2.405$; $p = .025$). However, no differences were found between NS-control and MS animals during the EPM (Fig. 2.3-2F and G).

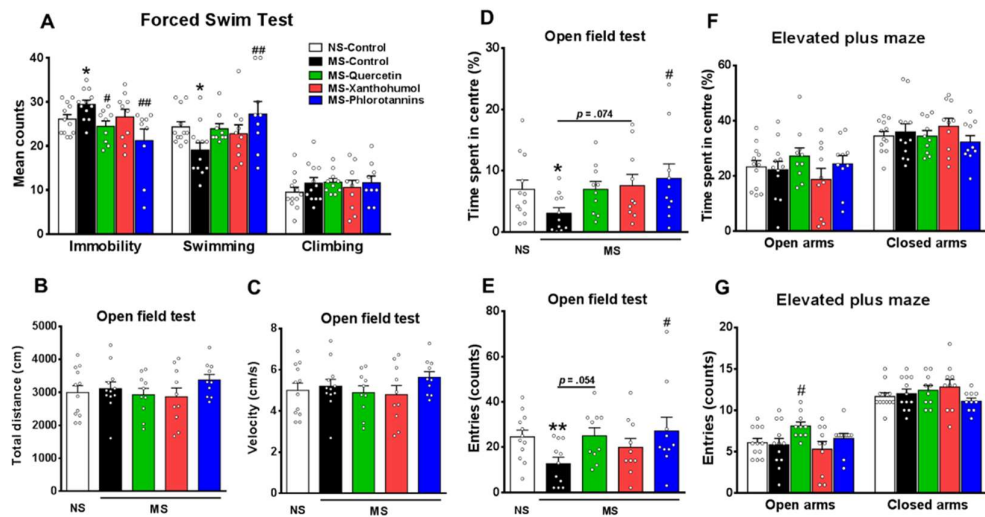


Figure 2.3-2 Treatment with polyphenols induced antidepressant- and anxiolytic-like effects in MS rats. (A) MS-induced increased immobility in the FST is prevented through treatment with quercetin and phlorotannins, while reduced swimming time caused by MS is reversed only by phlorotannins treatment. (B – C) Polyphenolic diets nor MS produced changes in locomotor activity. (D – E) Phlorotannins treatment produced a significant increase in the time spent in centre and number of centre entries when is compared to MS-control group in the OFT. (F – G) However, MS animals did not show anxiety-like behaviours in the EPM. Results are expressed as the mean \pm SEM (* $p < 0.05$; ** $p < 0.01$ versus ‘vehicle’ groups; # $p < 0.05$; ## $p < 0.01$ versus ‘CORT’ groups).

2.3.4.3 Xanthohumol prevented the exacerbated corticosterone production in MS rats after acute stress

To determine the role of the HPA axis in MS-induced depressive- and anxiety-like behaviours, the concentration of corticosterone in plasma was measured at different

time points after an acute stress (Fig. 2.3-3A). Indeed, the corticosterone production was close to being statistically increased in MS-control relative to the NS-control group as revealed by the area under the curve (AUC) of corticosterone response ($t_{20} = -1.949$; $p = .065$) (Fig. 2.3-3B). Interestingly, dietary intervention with xanthohumol in MS animals induced a significant reduction in corticosterone AUC compared to the MS-control group ($F_{3,34} = 3.827$; $p = .010$) (Fig. 2.3-3B). In addition, all polyphenolic treatments induced lower baseline levels of plasma corticosterone compared to MS-control group ($F_{3,36} = 3.979$; quercetin $p = .080$; xanthohumol $p = .006$; phlorotannins $p = .011$).

MS-induced plasma BDNF reduction was reversed by xanthohumol treatment

BDNF is a critical modulator of neuroplasticity and survival, abundant in the brain and periphery, including serum and plasma (Lee and Kim, 2010). Preclinical and clinical studies have demonstrated that chronic stress and depressive status reduces BDNF expression (Russo-Neustadt et al., 2001; Gonul et al., 2005). Indeed, MS rats showed lower levels of plasma BDNF compared to NS animals ($t_{19} = 2.672$; $p = .015$), and this effect was significantly prevented by xanthohumol treatment ($F_{3,36} = 1.748$; $p = .047$) (Fig. 2.3-3D).

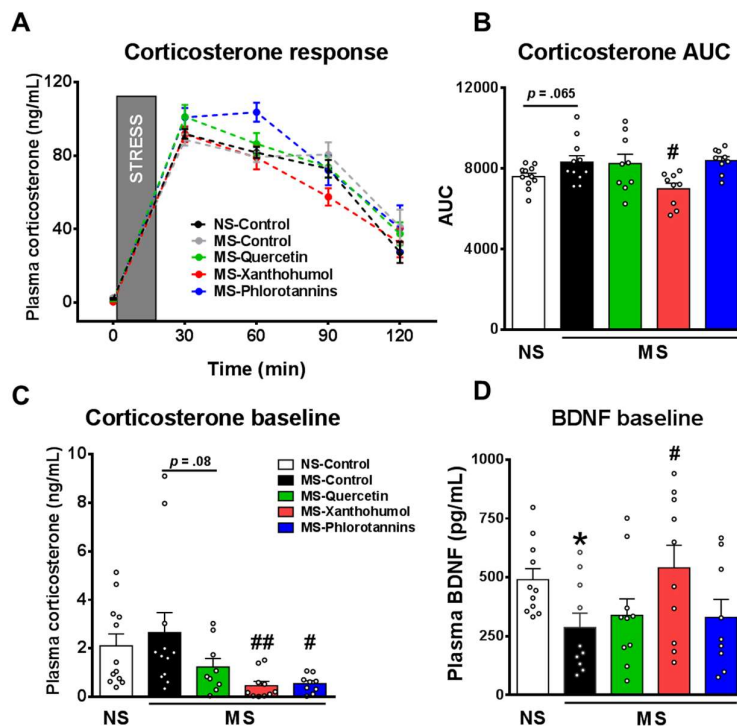


Figure 2.3-3 Xanthohumol treatment prevented corticosterone elevation and BDNF reduction in MS rats. (A) Corticosterone levels in plasma rise after rats are exposed to an acute stress. **(B)** MS-induced increase in corticosterone release is abolished by treatment with xanthohumol. **(C)** Baseline levels of corticosterone of rats treated with xanthohumol and phlorotannins are significantly lower compared to MS animals. **(D)** Rats treated with xanthohumol displayed higher levels of plasma BDNF compared to the MS-control group. Plasma corticosterone was determined using ELISA, and BDNF determination was performed with MSD system. Results are expressed as the mean \pm SEM (* $p < 0.05$ versus 'vehicle' groups; # $p < 0.05$; ## $p < 0.01$ versus 'CORT' groups).

2.3.4.4 MS induced decreased levels of DA and 5-HIAA in brainstem

To further determine the effects of early life stress on neurochemistry, and its potential implication on the antidepressant and anxiolytic effects of polyphenols, monoamine neurotransmitter concentration was measured in the brainstem. MS produced a significant reduction of DA and 5-HIAA levels ($t_{20} = 6.121$; $p = .000$ and $t_{22} = 3.934$; $p = .001$ respectively) (Fig. 2.3-4B and 2.3-D), reduced 5-HT turnover ($t_{21} = 3.519$; $p = .002$) (Fig. 2.3-4C), and increased DA turnover ($t_{22} = -2.153$; $p = .047$) (Fig. 2.3-4F). In contrast, treatment with phlorotannins increased the levels of NA and 5-HT compared to the MS-control group (Fig. 2.3-4A and G).

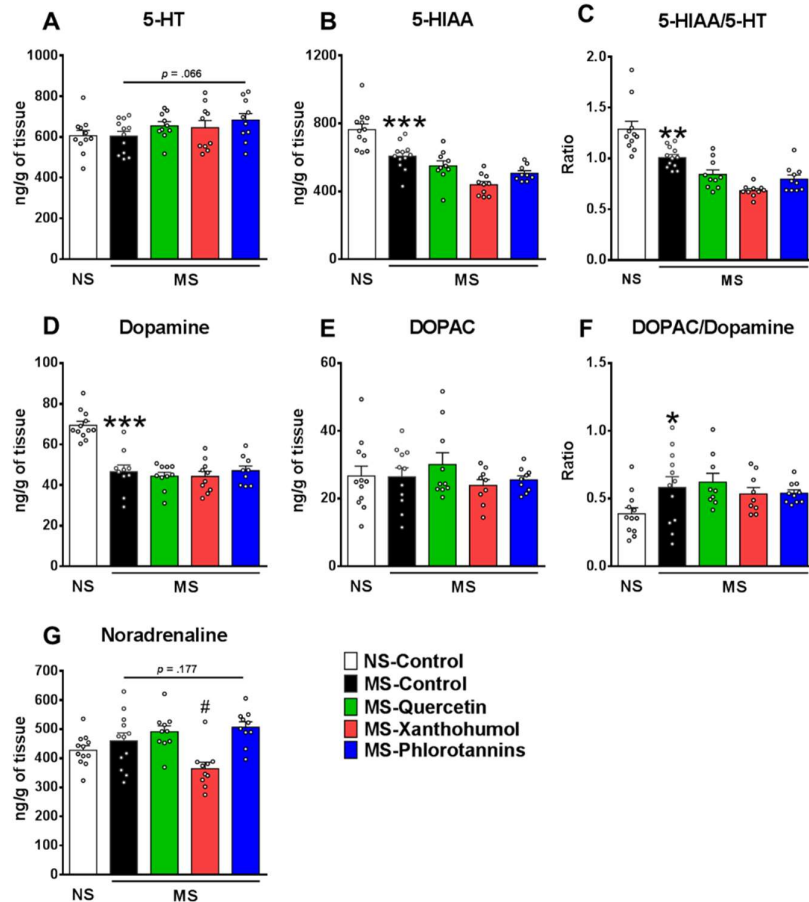


Figure 2.3-4 Polyphenols did not prevent MS-induced reductions in brainstem dopamine and 5-HIAA. Monoamine neurotransmitters were measured in the brainstem via HPLC. MS animals show depleted concentrations of dopamine and 5-HIAA compared to NS-control rats. Phlorotannins diet intervention exerted an increase of noradrenaline and 5-HT MS animals, although not significant. Results are expressed as the mean \pm SEM (* p < 0.05; ** p < 0.01; *** p < 0.001 versus 'vehicle' groups; # p < 0.05 versus 'CORT' groups).

2.3.4.5 MS and dietary treatments induced changes in gut bacterial diversity

To define whether the experimental treatments also altered gut microbiota diversity and bacterial abundance, α - and β -diversity analyses were performed. Although no differences in richness were found using the Chao1 α -diversity metric (Fig. 2.3-5A), Shannon entropy and Simpson index both indicate that MS rats treated with phlorotannins showed reduced diversity within this group compared to the MS-control experimental group (p = .072 and p < .05, respectively) (Fig. 2.3-5B and C). In other words, while the total estimated amount of OTUs did not differ, the microbial ecosystem of animals treated with phlorotannins were distributed less evenly. On

the other hand, principal component analysis (PCA) to measure the diversity among groups, indicated that MS and NS control groups were significantly different from each other ($F_{4,47} = 2.012$; $p = .046$) (Fig. 2.3-5D). In addition, treatments with quercetin, xanthohumol and phlorotannins also produced significant changes in β -diversity in terms of Aitchison distance compared to MS-control group ($F_{4,47} = 2.012$; $p = .004$; $p = .045$; $p = .046$, respectively) (Fig. 2.3-5D).

2.3.4.6 Changes in the gut microbiota composition correlated with MS status and polyphenolic diets

Alteration of the gut microbiota composition has been associated with different mental disorders, including major depression and other stress-related psychiatric disorders (Cryan and Dinan, 2012). Thus, we examined the differences in the gut microbiota composition of maternally separated rats. Significant differences in terms of the relative abundance between MS-control and NS-control animals were found in 5 specific bacteria based on effect size (*Streptococcus*; *Ruminococcus*; *Parabacteroides*; *Rothia*; *Christensenellaceae*; $q < .1$) (Fig. 2.3-5E). On the other hand, dietary interventions with quercetin and xanthohumol in MS rats induced significant changes in the abundance of other bacteria genera when compared to the MS-control group. Specifically, quercetin produced a significant increase of *Enterorhabdus* ($q < .1$), while xanthohumol exerted changes in the abundance of *Asteroplasma*, *Lachnospiraceae*, and *Coproccoccus* ($q < .1$) (Fig. 2.3-5E).

2.3.4.7 Treatment with phlorotannins and xanthohumol restore MS-induced changes in bacteria associated with microbiota-gut-brain pathways

To investigate the implications of MS-induced changes in gut microbiota composition on metabolic pathways associated with the microbiota-gut-brain axis, we performed a functional prediction based in previously described GBMs (Valles-Colomer et al., 2019). MS significantly changed the abundance of bacteria linked to 8 GBMs in terms of effect size, including tryptophan degradation, quinolinic acid metabolism, nitric oxide metabolism, and p-cresol synthesis compared to NS-control group ($q < .1$) (Fig. 2.3-5F). Intriguingly, although quercetin did not alter any relevant bacteria, xanthohumol and phlorotannins treatment restored most of the changes produced by MS in these bacteria.

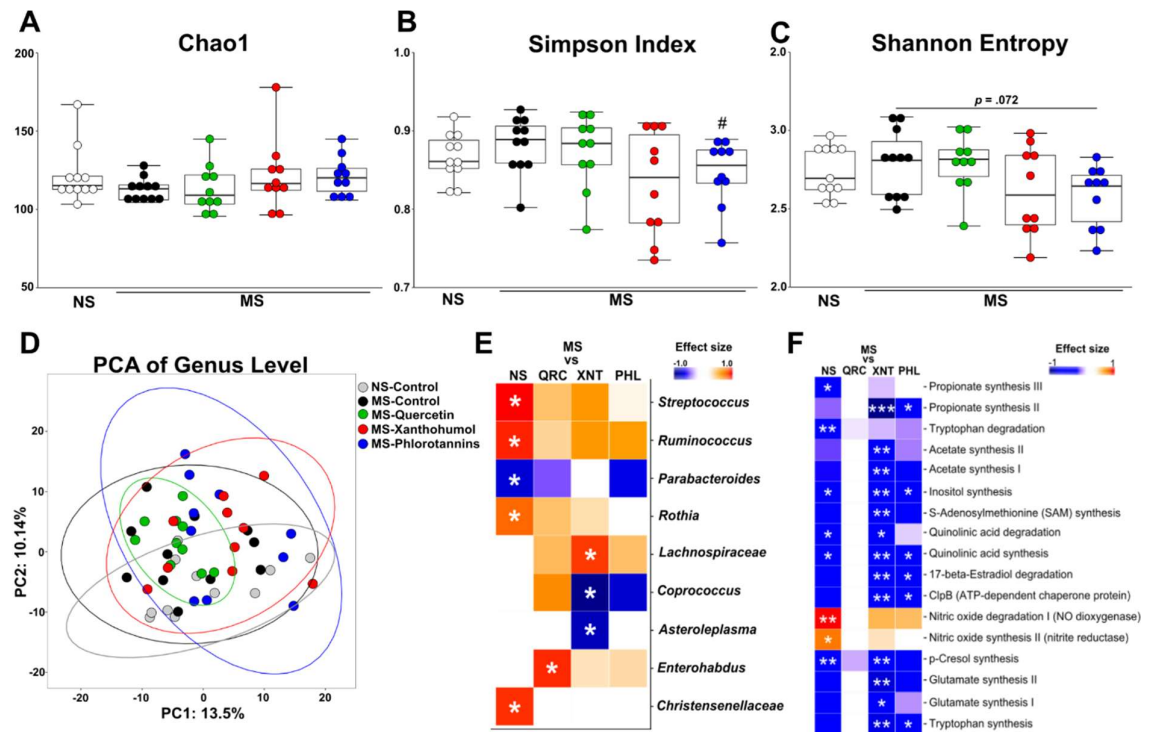


Figure 2.3-5 MS and polyphenolic diets induced significant changes in gut microbiota composition and diversity. (A – C) Chao1, Simpson index and Shannon entropy were used as estimators of bacterial α -diversity. (D) Principal component analysis of genus level was performed to estimate the β -diversity between experimental groups. (E) Bacterial abundances were significantly altered in MS rats in terms of effect size ($q < .1$). In contrast, xanthohumol and quercetin changed other bacteria compared to MS animals. (F) Functional prediction of GBMs was utilised to detect potential microbiota-gut-brain pathways affected by MS or dietary treatments. Colours represent effect size, only microbiome features found to be significantly different in at least one comparison are shown (* $q < .1$; ** $q < .05$; *** $q < .01$ vs MS-control group).

2.3.4.8 Xanthohumol prevented MS-induced reduction of intestinal SCFAs

To determine whether the observed changes in the gastrointestinal microbiota composition and diversity correlate with alteration in SCFA production, the levels of acetate, propionate, butyrate, valerate were determined in caecal content (Fig. 2.3-6). Interestingly, maternal separation induced a significant reduction of acetate ($t_{22} = 2.409$; $p = .025$), propionate ($t_{22} = 2.988$; $p = .01$), isobutyrate ($t_{21} = 3.354$; $p = .006$), isovalerate ($t_{21} = 2.779$; $p = .016$), total SCFAs ($t_{21} = 2.228$; $p = .037$), and total BCFA

($t_{21} = 3.181$; $p = .008$). In contrast, phlorotannin treatment significantly reversed the MS-induced propionate reduction ($F_{3,38} = 4.646$; $p = .022$), and exerted positive effects on isobutyrate, valerate and total levels of BCFAs.

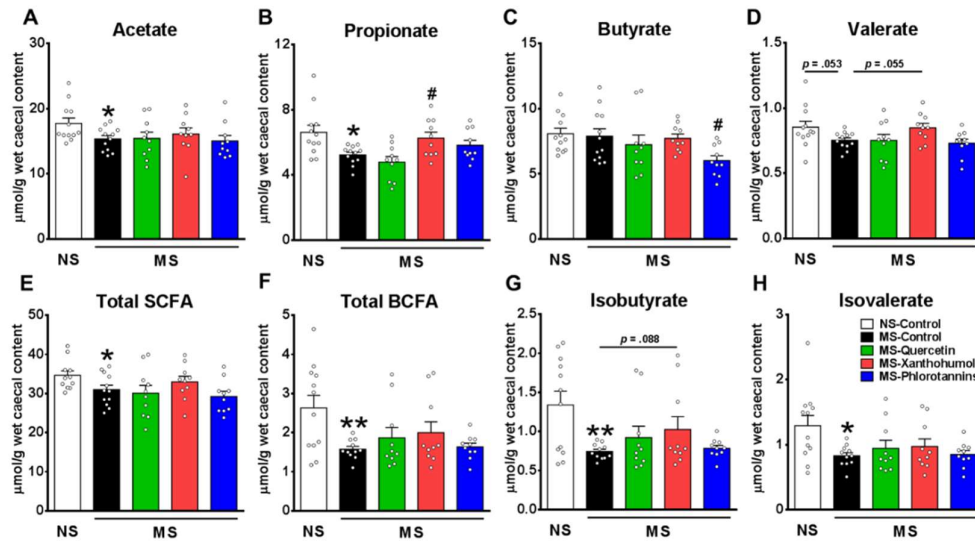


Figure 2.3-6 MS rats exhibited lower levels of gut microbiota-derived metabolites (A – H). MS induced significant reduction of gut microbiota-derived metabolites including acetate, propionate, isobutyrate and isovalerate, as well as decreased total short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA). Xanthohumol treatment ameliorated MS-induced propionate decrease and tends to improve isobutyrate and valerate levels. Fatty acid determination was performed through HPLC in caecal content. Results are expressed as the mean \pm SEM (* $p < 0.05$; ** $p < 0.01$ versus 'vehicle' groups; # $p < 0.05$ versus 'CORT' groups).

2.3.5 Discussion

There has been increasing attention given to the potential of nutritional approaches to ameliorate the effects of stress (Lakhan and Vieira, 2008; Marzola et al., 2013; Rechenberg and Humphries, 2013). In the present study, we tested different naturally-derived polyphenols as potential therapeutic strategies for depression and anxiety associated with early life trauma. Indeed, the polyphenols quercetin, xanthohumol and phlorotannins exert varying degrees of antidepressant- and anxiolytic-like responses in rats subjected to MS. Moreover, dietary interventions also modified gut microbial composition and diversity, suggesting that their therapeutic effects could be associated with the microbiota-gut-brain axis.

The MS rat is an excellent model to study the negative effects of early life stress on brain function and structure, which are associated with the development of depression and anxiety (O'Mahony et al., 2011; Vetulani, 2013). MS in rats induce a robust depressive-like phenotype in adult animals, including changes in gut microbiota, dysregulation of the HPA axis, and an imbalance in neurotransmitter levels (Daniels et al., 2004; Aisa et al., 2007; O'Mahony et al., 2009; Desbonnet et al., 2010; Liao et al., 2019). Furthermore, we demonstrated that all polyphenolic treatments tend to reverse these depressive-like behaviours. In particular, the phlorotannin-enriched diet produced a significant improvement in immobility and swimming behaviour in the FST compared to the MS-control group. Although the effect of polyphenols has been recently investigated in animal models of stress (Kwatra et al., 2016; Yang et al., 2017; Samad et al., 2018), to our knowledge there is no data on the effects of dietary intervention with polyphenols in animal models of early life stress *per se*.

Regarding anxiety, quercetin administration exerted a significant anxiolytic effect in MS animals, resulting in an increase in the number of entries into the open arms of the EPM. Similarly, quercetin- and xanthohumol-enriched diets tend to induce anxiolytic effects in the OFT, while phlorotannin treatment revealed a significant improvement. Although the concept of a potential therapeutic effect of polyphenols in animal models of stress is not completely new (Anjaneyulu et al., 2003; Hurley et

al., 2014; Aubry et al., 2019), the neurobehavioural effects of polyphenols in a model of early-life stress have not to our knowledge been examined previously.

We further investigated the role of the HPA axis in the therapeutic effects of polyphenol administration. Accumulated lines of evidence indicate that depressive or chronically stressed patients have an over activated HPA axis (Pariante and Lightman, 2008; Keller et al., 2017). Similarly, animals subjected to chronic stress possess a dysregulated HPA axis and increased baseline levels of glucocorticoids (O'Mahony et al., 2011; Uschold-Schmidt et al., 2012). Indeed, we demonstrated that the dietary intervention with xanthohumol significantly reduced the exacerbated production of corticosterone in MS animals.

Next, we demonstrated that treatment with xanthohumol prevented the MS-induced reduction in plasma BDNF. BDNF has strongly been implicated in antidepressant activity, and plasma BDNF has been shown to reflect aspects of that centrally and to be a biomarker of antidepressant effect (Sen et al., 2008; Lee and Kim, 2010; Woelfer et al., 2019). In addition, a positive correlation of BDNF levels between blood and brain has been demonstrated in rats (Karege et al., 2002; Sartorius et al., 2009; Harris et al., 2016), and a substantial amount of the circulating BDNF has been proposed to originate from the CNS itself (Dawood et al., 2007; Krabbe et al., 2007; Rasmussen et al., 2009). Although the possible pathways involved in BDNF rescue must be further investigated, it is tempting to speculate that the positive effects of xanthohumol on behaviour could be partly mediated by normalising BDNF expression.

The relationship between stress and the gut microbiota is gaining a lot of attention (Foster et al., 2017; Bastiaanssen et al., 2020). Additionally, we have demonstrated that MS is able to induce strong changes to the gut microbiota in terms of composition and diversity which is in line with previous reports (O'Mahony et al., 2009; Moussaoui et al., 2017). Although, we did not detect changes at the α -diversity level, analysis of β -diversity revealed that MS groups treated with polyphenols differ from the MS control group. We followed this up by assessing differential abundance of bacterial genera between the treatment groups. Notably, only xanthohumol and quercetin treatments produced significant changes in certain bacterial genera in MS

rats, suggesting that some polyphenol-enriched diets have the potential to modify bacterial composition in the gastrointestinal system. Several studies have demonstrated the capacity of polyphenolic intake to shape the gut microbiota (Etxeberria et al., 2013; Ozdal et al., 2016). The fact that all types of polyphenol intake were found to alter β -diversity compared to MS control, but only xanthohumol and quercetin yielded differences in the abundances of specific genera may suggest that polyphenols induce a general shift in the microbial composition, which may be indicative of a change in functionality in the microbiome.

Therefore, we performed a functional prediction of the gut metagenome and used this to infer the abundance of GBMs, metabolic modules that are involved in the microbiota-gut-brain axis (Valles-Colomer et al., 2019). Indeed, the analysis predicted that MS is able to increase the abundance of GBMs associated with the modulation of several pathways altered in depression and other neuropsychiatric disorders, including metabolism of tryptophan (Curzon and Bridges, 1970; Oxenkrug, 2010), inositol (Coupland et al., 2005), p-cresol (Persico and Napolioni, 2013), quinolinic acid (Steiner et al., 2011), nitric oxide (Dhir and Kulkarni, 2011), and glutamate (Sanacora et al., 2012; Murrough et al., 2017). Interestingly, treatment with xanthohumol and phlorotannins reversed these predicted MS-induced changes, suggesting that restoration of these GBMs may partially explain their positive effects in behaviour. An important limitation due to the nature of 16S sequencing is that functional analysis can only be inferential. Future metabolomics-based studies should address this experimentally.

In addition, our data revealed that MS rats exhibited decreased production of SCFAs compared with the NS-control group. We detected a significant reduction of acetate, propionate, isobutyrate, and isovalerate. The production of SCFAs is highly associated with certain bacterial populations in the gut, and there is common agreement surrounding the impact of SCFAs on human metabolism and health (Morrison and Preston, 2016). Indeed, it is widely accepted that SCFAs play a critical role in gut-microbiota-brain communication, and consequences for mental health and behaviour (Stilling et al., 2016; Dalile et al., 2019). A preclinical study showed that a depression-associated microbiota makeup can impact SCFA production (Kelly

et al., 2016), and that SCFAs can reverse the enduring effects of stress in a mouse model (van de Wouw et al., 2018). In our study, we demonstrated that treatment with xanthohumol specifically prevented the reduction of propionate in MS rats. Since the xanthohumol diet intervention induced acute changes in bacterial composition of the MS gut microbiota, we presume that the changes observed in propionate levels could be a product of improved microbial metabolism.

In conclusion, our present work confirmed that the naturally derived polyphenols xanthohumol, quercetin and phlorotannins can alleviate depressive- and anxiety-like behaviours in the rat MS model. We further found that treatment with xanthohumol prevented exacerbated production of corticosterone after acute stress in MS animals, and reversed MS-induced plasma BDNF depletion. In addition, our data revealed that MS-induced behavioural despair correlated with significant changes in bacterial composition and diversity, alteration of predicted microbiota-gut-brain pathways, and reduced SCFA production. Although all polyphenols caused changes in diversity, only xanthohumol induced significant changes in several bacterial taxa and prevented the reduction of propionate in MS rats. Taken together, our findings present evidence of the therapeutic properties of polyphenols and provide a novel insight into the potential mechanisms underlying their antidepressant effect.

2.4 Prebiotic administration modulates gut microbiota and faecal short-chain fatty acid concentrations but does not prevent chronic intermittent hypoxia-induced apnoea and hypertension in adult rats

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2.4.1 Abstract

Background: Evidence is accruing to suggest that microbiota-gut-brain signalling plays a regulatory role in cardiorespiratory physiology. Chronic intermittent hypoxia (CIH), modelling human sleep apnoea, affects gut microbiota composition and elicits cardiorespiratory morbidity. We investigated if treatment with prebiotic fibres, promoting the expansion of beneficial microbes, ameliorates cardiorespiratory dysfunction in CIH-exposed rats.

Methods: Adult male rats were exposed to CIH (96 cycles/day, 6.0% O₂ at nadir) for 14 consecutive days with and without prebiotic supplementation (fructo- and galacto-oligosaccharides) beginning two weeks prior to gas exposures.

Findings: CIH increased apnoea index and caused cardiac autonomic imbalance and hypertension. CIH exposure had modest effects on the gut microbiota, decreasing the relative abundance of *Lactobacillus rhamnosus*, but had no effect on microbial functional characteristics. Faecal short-chain fatty acid (SCFA) concentrations, plasma and brainstem pro-inflammatory cytokine concentrations and brainstem neurochemistry were unaffected by exposure to CIH. Prebiotic administration modulated gut microbiota composition and diversity, altering gut-metabolic (GMMs) and gut-brain (GBMs) modules and increased faecal acetic and propionic acid concentrations, but did not prevent adverse CIH-induced cardiorespiratory phenotypes.

Interpretation: CIH-induced cardiorespiratory dysfunction is not critically dependent upon decreased gut SCFA concentrations. Prebiotic-related boosting of SCFAs and modulation of GBMs and GMMs were not sufficient to prevent CIH-induced apnoea and hypertension in our model. Our findings reveal that although multiple correlations were evident between bacterial species and blood pressure, it is improbable that the gut microbiota play a critical role in the development of CIH-induced cardiorespiratory and autonomic dysfunction. Interestingly, prebiotic administration altered ventilatory responses to hypercapnic chemostimulation. Our findings are relevant to human sleep-disordered breathing and contribute to an

emerging interest in the potential manipulation of the gut microbiota as an adjunctive therapy for human cardiorespiratory disease.

2.4.2 Introduction

Obstructive sleep apnoea (OSA), the most common form of sleep-disordered breathing (SDB), is recognised as a major worldwide health crisis with devastating consequences for integrative body systems (Garvey et al., 2015). OSA is characterised by repetitive collapse of the pharyngeal airway during sleep, with episodic oxygen fluctuations culminating in recurrent exposure to chronic intermittent hypoxia (CIH). It is now apparent that exposure to CIH has adverse effects on the cardiorespiratory control network and is recognised as a major driver of OSA-related morbidities (Prabhakar et al., 2007; Julien et al., 2008; Edge and O'Halloran, 2015; O'Halloran, 2016; Iturriaga et al., 2017; Laouafa et al., 2017; Elliot-Portal et al., 2018).

Studies have recurrently implicated the carotid bodies, the dominant peripheral oxygen sensors, in the manifestation of CIH-induced cardiorespiratory dysfunction (Prabhakar et al., 2007; Peng et al., 2011; Iturriaga et al., 2015; Del Rio et al., 2016; Iturriaga et al., 2017). However, exposure to CIH elicits cardiorespiratory and autonomic disturbances in guinea-pigs with hypoxia-insensitive carotid bodies (Docio et al., 2018; Lucking et al., 2018), revealing that sites beyond the carotid bodies can contribute to the manifestation of CIH-induced cardiorespiratory and autonomic disturbances. It is known that CIH-induced plasticity also occurs at other key sites of the cardiorespiratory control circuit, including the nucleus tractus solitarius (NTS), pre-Bötzinger complex, ponto-medullary network and paraventricular nucleus of the hypothalamus (Veasey et al., 2004; Almado et al., 2012; Moraes et al., 2013; Garcia et al., 2016; Garcia et al., 2017; Li et al., 2018). More recently, studies have described effects of CIH on other peripheral sites including the gut microbiota (Moreno-Indias et al., 2015; Moreno-Indias et al., 2016; Lucking et al., 2018; AlMarabeh et al., 2019; O'Neill et al., 2019).

The microbiota-gut-brain axis plays a critical regulatory role in physiological systems. Dysregulated microbiota-gut-brain axis signalling affects homeostatic neurocontrol networks manifesting in pathophysiological behaviours and brain functions (Golubeva et al., 2015; Kelly et al., 2016; Dinan and Cryan, 2017). Recent studies extend this concept to cardiorespiratory control (Lucking et al., 2018; O'Connor et al., 2019). There is considerable interest in the modulatory role of the gut microbiota and gut microbiota metabolites, particularly short-chain fatty acids (SCFAs), in cardiovascular and autonomic function (Durgan et al., 2016; Ganesh et al., 2018; Kim et al., 2018; Meng et al., 2019). Proliferation of lactate-producing, as well as diminished butyrate- and acetate-producing taxa is evident in hypertensive models (Yang et al., 2015; Durgan et al., 2016; Adnan et al., 2017; Kim et al., 2018). Hypertensive donor faeces transferred to normotensive animals leads to the development of hypertension in recipient animals (Durgan et al., 2016; Adnan et al., 2017; Toral et al., 2019). Moreover, in a rat model of SDB, prebiotic administration stimulates the expansion of beneficial commensal microbiota augmenting several SCFA-producing taxa, restoring caecal acetate concentrations and preventing the establishment of hypertension (Ganesh et al., 2018). Chronic acetate administration into the caecum of OSA + high-fat diet (OSA+ HFD) rats prevents the development of high blood pressure (Ganesh et al., 2018). Additionally, butyrate treatment in angiotensin-II-induced hypertensive mice as well as spontaneously hypertensive rats prevented the establishment of hypertension (Kim et al., 2018; Robles-Vera et al., 2020).

In rat models, disruption of the gut microbiota using antibiotic administration, faecal microbiota transfer or pre-natal stress results in altered ventilatory responses to hypoxic and hypercapnic chemostimulation (Golubeva et al., 2015; O'Connor et al., 2019). Respiratory frequency response to hypercapnic chemostimulation correlated with altered bacterial genera in adult rats with antecedent pre-natal stress (Golubeva et al., 2015). Several genera, predominantly from the phylum Firmicutes correlated with brainstem neuromodulators crucial in the control of breathing (O'Connor et al.,

2019). Exposure to CIH dysregulates cardiorespiratory control in guinea pigs resulting in aberrant phenotypes including altered autonomic control of heart rate, decreased respiratory variability and prevalence of protective sighs and brainstem noradrenaline concentrations, as well as disturbed gut microbiota indicating that aberrant gut microbiota may at least partly contribute to cardiorespiratory and autonomic malaise in CIH-exposed guinea pigs (Lucking et al., 2018).

Collectively, these studies highlight a contributory role of perturbations to microbiota-gut-brain axis signalling in the manifestation of CIH-induced cardiorespiratory dysfunction, of relevance to OSA. There is a growing interest in developing strategies to manipulate the microbiota as a potential therapeutic intervention in the treatment of cardiorespiratory disease. Rodent and human studies have revealed that prebiotic administration has positive impacts on brain neurochemistry and functions (Savignac et al., 2013; Burokas et al., 2017; Dinan and Cryan, 2017; Mika et al., 2017). Moreover, prebiotic feeding prevented the development of hypertension in a rat model of OSA (Ganesh et al., 2018). Therefore, we assessed cardiorespiratory physiology and gut microbiota composition and diversity in adult rats following exposure to normoxia (Sham) or CIH. We hypothesised that there would be evidence of cardiorespiratory and autonomic dysfunction and altered gut microbiota in CIH-exposed rats. We examined the effects of prebiotic fibre supplementation to test the hypothesis that manipulation of the gut microbiota ameliorates or prevents the deleterious effects of exposure to CIH on cardiorespiratory physiology. We performed whole-genome shotgun sequencing in an attempt to identify microbial patterns that underscore cardiorespiratory homeostasis and dysfunction.

2.4.3 Materials and Methods

2.4.3.1 Ethical approval

Procedures on live animals were performed in accordance with European directive 2010/63/EU under authorisation from the Government of Ireland Department of Health (B100/4498) and Health Products Regulatory Authority (AE19130/P070). Ethical approval was obtained from University College Cork (AEEC #2013/035; #2017/023) and procedures were carried out in accordance with guidelines laid down by University College Cork's Animal Welfare Body.

2.4.3.2 Experimental animals

Eight- to ten-week old adult male Sprague Dawley rats (n=72; purchased from Envigo, UK) were housed as age-matched pairs in standard rat cages. Rodents had *ad libitum* access to standard rat chow and were housed under a 12-hr light: 12-hr dark cycle.

2.4.3.3 Prebiotic administration

Eight-week old rats (n=24) were randomly allocated to receive prebiotic fibres in the drinking water (PREB; galactooligosaccharides and fructooligosaccharides) with *ad libitum* access for 4-weeks to promote the growth of beneficial host microbiota as previously described (Savignac et al., 2013; Gronier et al., 2018; Yang et al., 2018). After 2 weeks of PREB treatment, a subset of rats were exposed to CIH (see section 2.4) for the final 2 weeks creating two groups: Sham+PREB (n=12) and CIH+PREB (n=12).

2.4.3.4 Chronic intermittent hypoxia rat model

Ten-week old rats (n=48) were randomly assigned to one of two groups, each receiving vehicle (VEH): Sham+VEH (n=24) and CIH+VEH (n=24). CIH exposed rats were placed in chambers wherein ambient oxygen concentration was tightly

regulated using a dynamic oxygen/nitrogen controller (Oxycycler™; Biospherix, New York, NY, USA). CIH exposure was comprised of 96 cycles of 90 secs of exposure to hypoxia (nadir, $\text{FiO}_2 = 0.06$, balance N_2) and 180 secs of exposure to normoxia ($\text{FiO}_2 = 0.21$; balance N_2), over 8 hours during the light phase for 14 consecutive days. Animals were studied on the day subsequent to the last day of CIH exposure. Concurrently, rats assigned to the Sham group were exposed to room air (normoxia) in the same room with similar environmental cues for the duration of the study.

2.4.3.5 Assessment of respiratory flow in rats during quiet rest

2.4.3.5.1 Whole-body plethysmography

During quiet rest, whole-body plethysmography (DSI, St. Paul, Minnesota, USA) was used to record respiratory flow signals during quiet rest. Animals were placed into custom plethysmograph chambers (601-1427-001 PN, DSI) with a room air flow rate maintained at 3l/min. Animals were allowed to acclimate for 30-90 minutes to encourage habituation to the new surroundings. Paired contemporaneous observations were performed during light hours in Sham+VEH (n=12) *versus* CIH+VEH (n=12) and subsequently Sham+PREB (n=12) *versus* CIH+PREB (n=12) using a pair of plethysmograph chambers.

2.4.3.5.2 Metabolic measurements

O_2 consumption (VO_2) and CO_2 production (VCO_2) were measured in rodents throughout the experimental protocol (O_2 and CO_2 analyser; AD Instruments, Colorado Springs, CO, USA) as previously described (Haouzi et al., 2009; Bavis et al., 2014; Lucking et al., 2018; O'Connor et al., 2019).

2.4.3.5.3 Experimental protocol

Once the acclimation period was complete and animals were confirmed to be at quiet rest, baseline parameters were recorded during a 10-15 minute steady-state normoxia period ($\text{FiO}_2 = 0.21$; balance N_2). This was followed by a 10 minute

poikilocapnic hypoxia challenge ($\text{FiO}_2=0.10$; balance N_2). Normoxia was subsequently restored in each chamber to re-establish stable baseline breathing. Thereafter, a second baseline period was recorded followed by a 10 minute hypercapnia challenge ($\text{FiCO}_2 = 0.05$; balance O_2). Subsequently, a third normoxic baseline period was recorded. Rats were then exposed to a 10 minute hypoxic hypercapnic challenge ($\text{FiO}_2 = 0.10$; $\text{FiCO}_2 = 0.05$, balance N_2).

2.4.3.5.4 Data analysis for whole-body plethysmography

Respiratory parameters including tidal volume (V_T), respiratory frequency (f_R), minute ventilation (V_I), expiratory time (T_e) and inspiratory time (T_i) were recorded on a breath-by-breath basis for analysis (FinePointe software Buxco Research Systems, Wilmington, NC, USA). Artefacts relating to animal movement and sniffing in respiratory flow recordings were omitted from analysis. A single baseline period during normoxia was determined by averaging the three baseline recording epochs to determine resting steady-state respiratory and metabolic parameters. Ventilatory and metabolic data were averaged and reported for the final 5 minutes of acute poikilocapnic hypoxia, hypercapnia and hypoxic hypercapnia allowing sufficient time for gas mixing in the custom plethysmograph chambers. Data are expressed as absolute change from baseline values. Respiratory flow recordings were assessed for the occurrence of augmented breaths (sighs) during normoxia, poikilocapnic hypoxia and hypercapnia, as well as the frequency of apnoea events (post-sigh and spontaneous apnoeas) during normoxia as previously described (Edge et al., 2012). The criterion for an apnoea was a pause in breathing greater than two consecutive missed breaths. Apnoea data are expressed as apnoea index (apnoea events per hour), combining post-sigh and spontaneous apnoeas. A sigh was defined as an augmented breath, double the amplitude of the average V_T . The frequency and amplitude of sighs were determined. Poincaré plots expressing breath-to-breath (BBn) *versus* subsequent breath-to-breath interval ($\text{BBn}+1$) were extrapolated allowing for determination of short- (SD1) and long-term (SD2) respiratory timing variability during steady-state baseline breathing. V_T , V_I , V_T/T_i , VO_2 and VCO_2 were normalised per 100g body mass.

2.4.3.6 Assessment of cardiorespiratory parameters under urethane anaesthesia

2.4.3.6.1 Surgical protocol and cardiorespiratory measures

Following whole-body plethysmography, cardiorespiratory parameters were assessed in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB rats (n=11-12 per group) under urethane anaesthesia (1.5 g/kg i.p.; 20% w/v) following isoflurane induction (5% by inhalation in room air). Throughout the surgical and experimental protocol, the depth of anaesthesia was carefully assessed by monitoring reflex responses to tail/paw pinch and the corneal reflex. Supplemental doses of anaesthetic were given as required. Rodents were placed in a supine position on a homeothermic blanket system (Harvard Apparatus, Holliston, MA, USA) and a rectal temperature probe and heating pad used to maintain core temperature at 37 °C. A mid-cervical tracheotomy was performed, followed by intravenous (i.v.) cannulation of the right jugular vein for infusion of supplemental anaesthetic and drugs. The carotid (n=22)/femoral artery (n=1) was cannulated for the recording of arterial blood pressure and the withdrawal of blood samples for arterial blood gas, pH and electrolyte analysis (i-STAT; Abbott Laboratories Ltd). Using a foot clip, arterial oxygen saturation (SaO₂; Starr Life Sciences, PA, USA) was determined and maintained above 95% via a bias flow of supplemental O₂ passing the tracheal cannula sourced from a gas mixing system (GSM-3 Gas Mixer; CWE Inc.). A pneumotachometer (Hans Rudolf) and a CO₂ analyser (microCapStar End-Tidal CO₂ analyser; CWE Inc., USA) were connected to the tracheal cannula to measure tracheal flow and end-tidal CO₂ (ETCO₂), respectively. Diaphragm electromyogram (EMG) activity was continuously measured using a concentric needle electrode (26G; Natus Manufacturing Ltd., Ireland). Signals were amplified (x5,000), filtered (band pass; 500–5000 Hz) and integrated (50 ms time constant; Neurolog system, Digitimer Ltd., UK). Data were digitised via a PowerLab-LabChart v7 (ADInstruments) data acquisition system.

2.4.3.6.2 Experimental protocol

An arterial blood sample was obtained from each animal following a 30 minute stabilisation period, after which, a minimum 10 minute baseline recording period was observed for assessment of baseline parameters ($\text{FiO}_2 = 0.25\text{-}0.40$; balance N_2). The rats were exposed to a poikilocapnic hypoxia challenge ($\text{FiO}_2 = 0.12$, balance N_2) for 5 minutes, followed by a recovery period. Animals were then exposed to a 5 minute hypoxic hypercapnic challenge ($\text{FiO}_2 = 0.12$, $\text{FiCO}_2 = 0.05$, balance N_2). Following a recovery period, sodium cyanide (NaCN ; $200\text{ }\mu\text{g/kg}$ i.v.) was administered to evoke carotid body-dependent increases in ventilation. After an adequate recovery period and removal of the pneumotachometer, a second arterial blood sample was taken. Next, the serotonin type 3 (5-HT_3) receptor agonist phenylbiguanide (PBG; $25\text{ }\mu\text{g/kg}$ i.v.) was administered to stimulate pulmonary vagal afferent C-fibres (Dutta and Deshpande, 2010; Lucking et al., 2018) eliciting the classic pulmonary chemoreflex. Successively, phenylephrine ($50\text{ }\mu\text{g/kg}$ i.v.), sodium nitroprusside ($50\text{ }\mu\text{g/kg}$ i.v.), atenolol (2 mg/kg i.v.), propranolol (1 mg/kg i.v.) and hexamethonium (25mg/kg i.v.) were administered to assess cardiovascular responses to pharmacological manipulation with sufficient recovery periods allowed between each pharmacological challenge. Animals were euthanised by urethane (i.v) overdose. One animal (Sham+PREB, $n=1$) presented with uncharacteristically poor ventilatory and cardiovascular responses throughout the experimental protocol; this animal was excluded from data analysis. In all animals, blood was collected, prepared in 3% Na_2EDTA (disodium salt dehydrate) and centrifuged. Plasma was snap frozen in liquid nitrogen for subsequent analysis of corticosterone and pro-inflammatory cytokine concentrations. Whole brains were removed, separated into pons and medulla oblongata, frozen in isopentane at $-80\text{ }^\circ\text{C}$ and stored at $-80\text{ }^\circ\text{C}$ until subsequent analysis by high-performance liquid chromatography. The lungs were removed and weighed and were allowed to air dry at 37°C for at least 48 hrs before being re-weighed to provide an index of pulmonary oedema. The caecum was removed, weighed and caecal contents were removed and snap frozen in liquid nitrogen for whole-genome shotgun sequencing. Faeces was removed from the colon for the assessment of SCFA concentrations by gas chromatography. The heart was removed,

and the right ventricle was separated from the left ventricle + septum and each were weighed separately.

2.4.3.6.3 Data analysis of cardiorespiratory parameters in anaesthetised rats

Baseline parameters were averaged over 10 minutes of stable recording and data are presented as absolute values. For cardiorespiratory and EMG responses to poikilocapnic hypoxia and hypoxic hypercapnia the average of the last minute of recordings was determined and data were compared with the 1 minute pre-challenge baseline. Data for drug challenges were averaged into 3 or 5 second bins and the peak cardiorespiratory responses to NaCN, PBG, phenylephrine, sodium nitroprusside, atenolol, propranolol and hexamethonium administration were determined and compared to the respective 1 minute pre-challenge baseline. Maximum apnoea and post-apnoea tachypnoea in response to PBG are expressed as the duration of the apnoea or tachypnoea period normalised in each trial to the average cycle duration determined during the 30 sec pre-challenge baseline period. All cardiorespiratory responses to chemostimulation and drug administration are expressed as percent change from the preceding baseline values.

2.4.3.7 Brainstem monoamine concentrations

2.4.3.7.1 High-performance liquid chromatography (HPLC) coupled to electrochemical detection for the measurement of brainstem monoamine concentrations

Pons (n=11-12/group) and medulla oblongata (n=11-12/group) tissues were sonicated in 1 ml of chilled mobile phase, spiked with 2ng/20µl of a N-methyl 5-HT (internal standard; Bandelin Sonolus HD 2070). Brainstem monoamine, precursor and metabolite concentrations were measured as previously described (Lucking et

al., 2018; O'Connor et al., 2019). Noradrenaline (NA), dopamine (DA), serotonin (5-HT), and monoamine metabolites and precursor, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and L-3,4 dihydroxyphenylalanine (L-DOPA) were identified by their characteristic retention times. This was determined by standard injections run at regular intervals during sample analysis.

2.4.3.7.2 Data analysis

Class-VP5 software was used to process chromatographs. Concentrations (ng/g) of monoamines, precursors and metabolites in each sample were determined using analyte:internal standard peak response ratios.

2.4.3.8 Plasma and brainstem pro-inflammatory cytokine concentrations

2.4.3.8.1 Brainstem tissue homogenisation and protein quantification

A separate cohort of rats (Sham, n=12; CIH, n=12) were euthanised by pentobarbitone (i.v.) overdose and whole brains were removed. The pons and medulla oblongata were separated from the brain, frozen in isopentane at -80°C and stored at -80°C until subsequent determination of brainstem cytokine concentrations. Pons and medulla oblongata tissue (Sham, n=12; CIH, n=12) were weighed and sonicated (1 ml per 100 mg of tissue) in radioimmunoprecipitation assay (RIPA buffer) (10X RIPA, deionised H₂O, 200Mm sodium fluoride, 100Mm, phenylmethylsulfonylfluoride (PMSF), 1X protease inhibitor cocktail and 1X phosphate inhibitor cocktail). Samples were centrifuged at 10,000 *g* for 15 minutes at 4 °C, to pellet membranes and nuclei. The protein concentration of each sample was determined using a bicinchoninic acid (BCA) protein quantification assay (Thermo Fisher Scientific) as per the manufacturer's instructions, at a dilution of 1:10.

2.4.3.8.2 Multiplex assay for measurement of plasma and brainstem pro-inflammatory cytokines

Concentrations of interleukin(IL)-1 β , IL-4, IL-5, IL-10, IL-13, interferon (IFN)- γ , keratinocyte chemoattractant/growth-related oncogene (KC/GRO) and tumor necrosis factor (TNF)- α were measured in plasma (all groups; n=11-12/group) as well as pons and medulla oblongata (Sham and CIH only; n=12 each group) supernatants by sandwich immunoassay methods using commercially available detection kits (V-Plex Pro-inflammatory Panel 2 (rat) kit; Meso Scale Discovery, Gaithersburg, USA) as per the manufacturer's instructions. For pons and medulla oblongata tissues, 100 μ g of protein sample was loaded per well as previously described (Lucking et al., 2018). Plates were read using QuickPlex SQ 120 imager and computer (Meso Scale Discovery).

2.4.3.9 Plasma corticosterone

Plasma samples were thawed and concentrations of corticosterone were determined using commercially available enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (ENZO Life Sciences, UK) using a spectrophotometer (SpectraMax M3, Molecular devices).

2.4.3.10 Microbiota composition and function

2.4.3.10.1 DNA extraction from caecal material

DNA was extracted from caecal material as previously described (Gough et al., 2018).

2.4.3.10.2 Whole-metagenome shotgun sequencing

Whole-metagenome shotgun libraries were prepared in accordance with the Nextera XT DNA Library Preparation Guide from Illumina with the exception that the tagmentation time was increased to 7 minutes. After indexing and clean-up of the PCR products as described in the protocol, each sample was run on an Agilent bioanalyser high sensitivity chip (Agilent) to determine the size range of the fragments obtained. The concentration of the samples was also determined at this point using a Qubit High Sensitivity Assay (Life-Sciences). Samples were then pooled equimolarly and the final concentration of the pooled library was determined by quantitative PCR using the Kapa Library Quantification kit for Illumina (Roche). The pooled library was then sequenced on the Illumina NextSeq using the 2 x150 High Output kit according to standard Illumina sequencing protocols.

2.4.3.10.3 Metagenomic bioinformatic analysis

Shotgun metagenomic sequence files (BCL, base calls) were converted to fastq format using bcl2fastq version 2.19. Forward and reverse fastq files were processed using KneadData version 0.7.2 from the Huttenhower bioBakery suite (McIver et al., 2018). A reference library was created to remove host DNA in Bowtie2 version 2.3.4 from the NCBI rat genome (*Rattus norvegicus*, GCF 000001895.5). Quality filtering was performed using the default setting (ex., phred=33) and trimming with Trimmomatic version 0.38-1. Resulting high quality paired-end reads for each sample were then concatenated in KneadData. Kraken2 version 2.0.7-beta was used for taxonomic classification with the standard database. Report files of taxonomic counts for each sample were merged into a single count file using a custom R script and ran in R version 3.5.2.

2.4.3.10.4 Functional annotation, gut-brain module and gut-metabolic module analysis

Humann2 was used to generate a table of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues. This table was aggregated into gut-brain modules (GBMs) and gut-metabolic modules (GMMs), pathways of functions performed by the gut

microbiome that have the potential to influence the host brain (Vieira-Silva et al., 2016; Valles-Colomer et al., 2019), using the omixer-rpmR library for R (Darzi et al., 2016).

2.4.3.11 Faecal short-chain fatty acid concentrations

SCFA analysis and extraction were carried out by MS-Omics (Vedbaek, Denmark) as follows. Faecal water was prepared by homogenising the faecal samples (approximately 100 mg) in ultrapure water (3µl/µg). Samples were then vortexed for 2 minutes followed by centrifugation (5 minutes, 30000 *g*, 5°C). The supernatant was transferred to a centrifuge filter and the filtered samples were used for analysis. The filtrate was acidified using hydrochloride acid, and deuterium labelled internal standards were added. All samples were analysed in a randomized order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m x 0.25 mm x 0.25 µm) installed in a gas chromatography (GC; 7890B, Agilent) coupled with a quadrupole detector (59977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISE software (Johnsen et al., 2017). Data are expressed as absolute concentration in mM.

2.4.3.12 Statistical analysis

Data (except microbiota data) was assessed for outliers, normal distribution and equality of variances using box-plots, Shapiro-Wilk test and Levene's test, respectively. In situations of normal distribution, data were statistically compared using independent samples *t*-test and two-way ANOVA followed by Fisher's least significant test for pairwise comparisons, where appropriate. In some instances, the assumptions of no significant outliers and homogeneity of variances were violated for the two-way ANOVA. When the assumption of normal distribution was violated, data were statistically compared using non-parametric Mann-Whitney *U* test and

non-parametric Kruskal-Wallis test followed by Mann-Whitney *U* test for pairwise comparisons, where appropriate. Statistical significance was assumed at $p < 0.05$. Bonferroni correction was applied to adjust for multiple comparisons with the exception of microbiota data. Statistical significance for multiple comparisons was accepted at $p < 0.05$ divided by the number of comparisons made.

When handling compositional data such as the microbiome taxonomic data, GBM and GMM, the compositionally appropriate centered-log ratio (clr) transformation was performed using the ALDEx2 R library in preparation of statistical testing (Aitchison, 1982). The ALDEx2 library was also used to test for differentially abundant features, using a pairwise implementation of the *aldex.ttest()* function to compare multiple groups. Benjamini-Hochberg (BH) adjustment procedure was applied with the false discovery rate (FDR) set at 10% to correct for multiple testing in the relative abundance microbiota data. The 2D principal component analysis (PCA) was constructed using the clr transformed values computed using the ALDEx2 (Fernandes et al., 2013) library in R (version 3.6.0) with Rstudio (version 1.1.453), as is appropriate for compositional data (Gloor et al., 2017) using recommended parameters and 1000 permutations. For correlation analysis between bacterial species and physiological parameters of interest, Hierarchical All-against-All association testing (HALLA) (Rahnavard et al., 2017) was used (version 0.8.7) with Spearman correlation as correlation metric, medoid as clustering method and $q < 0.1$ as threshold for significance. Microbiota data are expressed as median (IQR). All other data are expressed as mean \pm SD or displayed graphically as box and whisker plots (median, IQR and minimum to maximum values). SPSS v25 was used for all other statistical analysis. GraphPad Software v6 (GraphPad Software, San Diego, CA, USA) and R software environment were used to generate graphs. Adobe illustrator CS5 (v15) was used to edit figures.

2.4.4 Results

2.4.4.1 Body and tissue weights

CIH exposure and prebiotic administration had a significant effect on body weight (Diet*CIH, $F(1, 43) = 5.426$, $p=0.025$, $\eta^2=0.112$, Table 1). The combination of CIH+PREB, decreased body weight gain compared with CIH+VEH or Sham+PREB rats; Sham+PREB rats were also lighter than Sham+VEH rats. CIH exposure had no effect on caecum weight but as expected, prebiotic supplementation increased caecum weight. Differences between groups in normalised cardiac ventricle weights relate to changes in body weight (Supplementary table 1).

2.4.4.2 Baseline ventilation and metabolism in rats during quiet rest

CIH exposure did not affect the majority of respiratory parameters during normoxia. Prebiotic fibre supplementation increased baseline V_I , V_T and V_T/T_i in Sham+PREB and CIH+PREB compared with Sham+VEH and CIH+VEH rats, respectively (Table 2), but the differences related to body weight (Supplementary Table 1). CIH exposure had no effect on VCO_2 production; CIH+PREB rats had significantly increased VCO_2 production compared with CIH+VEH rats, but VCO_2 production in Sham+PREB rats was not different compared with Sham+VEH (Table 2). CIH exposure had no effect on V_I/VCO_2 (breathing as a function of metabolism), but prebiotic administration increased V_I/VCO_2 , however *post hoc* analysis revealed no difference between groups (Table 2). In summary, CIH exposure and prebiotic administration had modest effects on ventilation and metabolism during normoxia.

2.4.4.3 Respiratory timing variability, apnoeas and sighs during normoxia in rats during quiet rest

Assessments of short-term (SD1) and long-term (SD2) respiratory timing variability during normoxia did not reveal differences between groups ($p>0.05$; Fig. 2.4-1a-f). Apnoea index was significantly increased by CIH exposure ($\chi^2(3) = 9.284$, $p=0.026$, Fig. 2.4-1j), a consequence of alterations in spontaneous apnoea events; no statistically significant differences were evident in post-sigh apnoea events (Table 2). *Post hoc* analysis revealed that apnoea index was increased in CIH+PREB compared with Sham+PREB rats ($p=0.008$; Fig. 2.4-1j). The frequency of sighs was not affected

by CIH exposure or prebiotic administration ($p>0.05$; Fig. 2.4-1k). Sham+PREB had elevated sigh amplitude compared with Sham+VEH rats (Table 2). The major finding was that CIH exposure increased apnoea index during quiet breathing at rest (normoxia) and prebiotic administration did not prevent this aberrant phenotype.

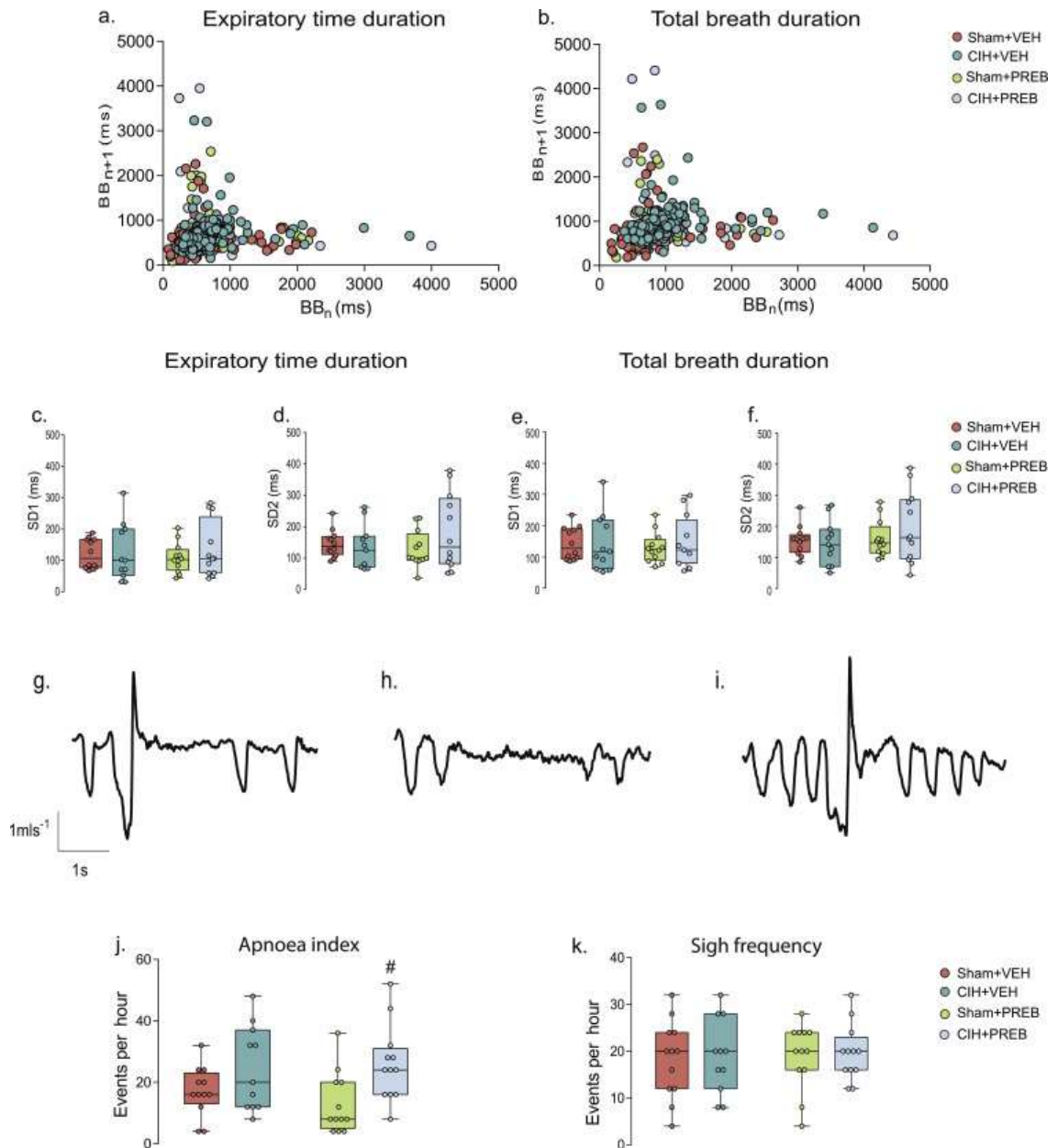


Fig 2.4-1. Poincaré plots of breath-to-breath (BB_n) and subsequent breath-to-breath ($BB_n + 1$) interval of expiratory duration (T_e ; a) and total breath duration (T_{tot} ; b) for Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. Group data for T_e short-term variability ($SD1$; c) and long-term variability ($SD2$; d) and T_{tot} $SD1$ (e) and $SD2$ (f) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB rats during normoxia. Representative respiratory flow traces (downward deflections represent inspiration)

illustrating a spontaneous sigh followed by an apnoea (g), a spontaneous apnoea (h) and a spontaneous sigh (i). Group data of apnoea index (j) and sigh frequency (k). CIH, chronic intermittent hypoxia; PREB, prebiotic; VEH, vehicle. Groups (c-f, j, k) are expressed as box and whisker plots (median, IQR and minimum to maximum values); n = 11–12. Groups were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test, where appropriate. Apnoea index was significantly affected by CIH exposure ($p = 0.026$; Fig. 1j). Assessments of respiratory timing variability and frequency of sighs were not different between groups ($p > 0.05$; Fig. 1a-f, 1k). # $p = 0.008$, CIH+PREB versus Sham+PREB.

2.4.4.4 Ventilatory and metabolic responsiveness to chemostimulation in rats during quiet rest

2.4.4.4.1 Ventilatory and metabolic responsiveness to hypoxic chemostimulation

No significant differences were evident in CIH+VEH compared with Sham+VEH rats.

CIH+PREB rats had decreased f_R , V_I , V_T/T_i and increased T_i and T_e compared with Sham+PREB rats (Table 3). T_i was decreased in Sham+PREB compared with Sham+VEH rats (Table 3). Sigh frequency and amplitude were not different in CIH+VEH compared with Sham+VEH rats, but CIH+PREB rats had less frequent but larger sighs compared with CIH+VEH rats. Sigh frequency was reduced in CIH+PREB compared with Sham+PREB rats (Table 3). The major observation was that prebiotic administration reduced the frequency of sighs during hypoxia in CIH-exposed rats.

2.4.4.4.2 Ventilatory and metabolic responsiveness to hypercapnic chemostimulation

CIH exposure elevated sigh frequency during hypercapnia, as such CIH+VEH rats had increased generation of sigh compared with Sham+VEH rats. Other respiratory and metabolic parameters were not different in CIH+VEH compared with Sham+VEH rats in response to hypercapnia (Table 3). Prebiotic administration in CIH-exposed rats elevated V_T/T_i compared with CIH+VEH rats. Interestingly, Sham+PREB rats had elevated ventilation (V_I) and increased drive to breathe (V_T/T_i) in response to hypercapnia compared with Sham+VEH rats, with no change in V_I/VCO_2 (Table 3). Furthermore, Sham+PREB rats had augmented sigh frequency and amplitude compared with Sham+VEH rats. There was no difference between CIH+PREB and Sham+PREB rats (Table 3). The major finding was that prebiotic fibre

supplementation increased sigh frequency and the ventilatory response to hypercapnia.

2.4.4.4.3 Ventilatory and metabolic responsiveness to hypoxic hypercapnic chemostimulation

No significant differences were evident in CIH+VEH compared with Sham+VEH rats. CIH+PREB rats had elevated V_I compared with CIH+VEH rats. Sham+PREB rats had an elevated ventilatory response to hypoxic hypercapnia compared with Sham+VEH rats, evident by increased V_I , V_T and V_T/T_I ; V_I/VCO_2 was not different between groups (Table 3). Furthermore, there was no apparent difference between Sham+PREB and CIH+PREB rats. The major observation was that prebiotic administration elevated the ventilatory response to hypoxic hypercapnia.

2.4.4.4.4 Baseline cardiorespiratory and blood gas parameters in anaesthetised rats

CIH exposure had no effect on respiration in the anaesthetised rat during baseline conditions (Table 4). V_I and V_T were increased in PREB+CIH rats compared with CIH-exposed rats (Table 4). CIH exposure significantly increased diastolic blood pressure (DBP) (CIH; $F(1,41) = 16.321$, $p < 0.0005$, $\eta^2 = 0.285$, Fig. 2.4-2b). As a consequence, mean arterial blood pressure (MAP) was elevated (CIH; $F(1, 41) = 17.485$, $p < 0.005$, $\eta^2 = 0.299$). *Post hoc* analysis revealed CIH+VEH had elevated blood pressure compared with Sham+VEH rats (DBP, $p = 0.006$, Fig. 2b; MAP, $p = 0.004$, Fig. 2.4-2a). DBP was not restored by prebiotic administration as CIH+PREB had elevated DBP compared with CIH+VEH rats ($p = 0.007$, Fig. 2.4-2b). There was no statistical difference evident in MAP between CIH+PREB compared with CIH+VEH ($p > 0.05$, Fig. 2.4-2a), and MAP was elevated in CIH+PREB compared with Sham+PREB rats ($p = 0.006$; Fig. 2.4-2a). CIH exposure or prebiotic administration had no effect on systolic blood pressure or heart rate ($p > 0.05$, Fig. 2.4-2c, 2.4-2d). CIH exposure had no effect on haematocrit and haemoglobin concentrations; Sham+PREB had reduced concentrations compared with Sham+VEH (Table 4).

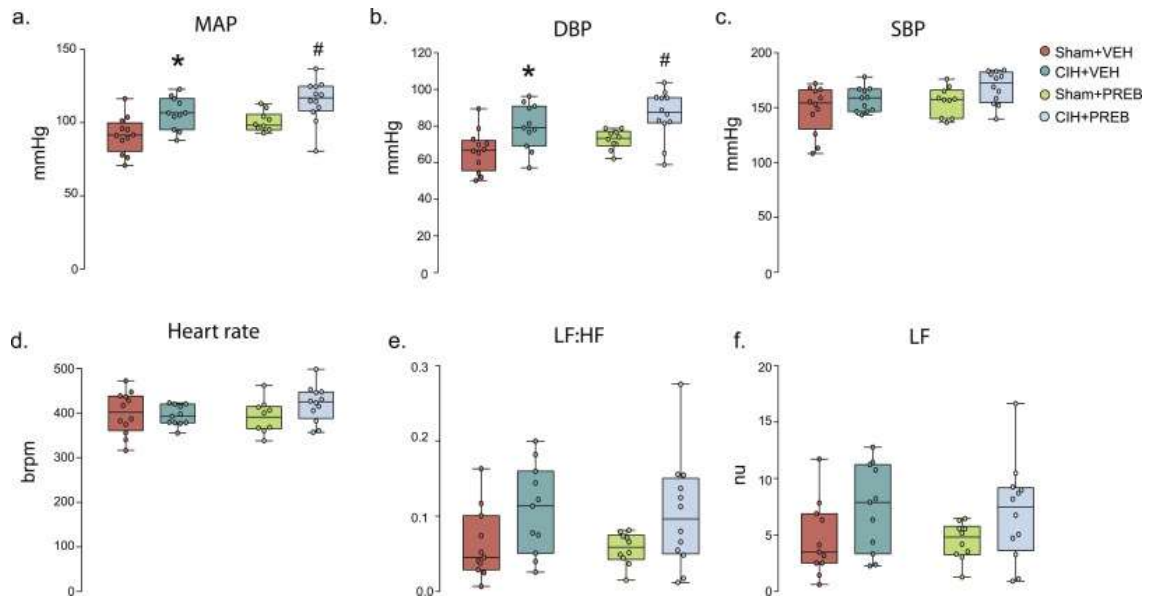


Fig. 2.4-2 Group data for MAP (a), DBP (b), SBP (c), heart rate (d), LF: HF (e) and LF (f) for Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. MAP, mean arterial blood pressure; DBP, diastolic blood pressure; SBP, systolic blood pressure; LF, low-frequency band; HF, high frequency band; CIH, chronic intermittent hypoxia; PREB, prebiotic; VEH, vehicle. Groups (a-f) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 10-12$ for all groups. Groups were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test, where appropriate. CIH significantly affected MAP, DBP, LF: HF and LF ($p < 0.005$, $p < 0.0005$, $p = 0.008$ and $p = 0.017$, respectively; Fig. 2a, 2b, 2e, 2f). There was no change in SBP or HR ($p > 0.05$, Fig. 2c, 2d). * $p = 0.004$, CIH+VEH versus Sham+VEH; # $p < 0.01$, CIH+PREB versus Sham+PREB.

CIH exposure increased the low-frequency band (LF) (CIH; ν , $F(1, 40) = 6.170$, $p = 0.017$, $\eta^2 = 0.134$, Fig. 2.4-2 f; %, $F(1, 40) = 6.723$, $p = 0.013$, $\eta^2 = 0.144$, Table 5) and decreased the high-frequency band (HF; μ) (CIH, $F(1, 40) = 1.159$, $p = 0.014$, $\eta^2 = 0.142$, Table 5) elevating the LF: HF ratio (CIH, $F(1, 40) = 7.748$, $p = 0.008$, $\eta^2 = 0.162$, Fig. 2.4-2 e) during steady-state baseline recordings, indicating sympathetic dominance. LF:HF was increased in CIH+VEH compared with Sham+VEH rats ($p = 0.059$, Fig. 2.2-4 e). There was no difference in CIH+PREB compared with CIH+VEH rats ($p > 0.05$), however CIH+PREB rats had elevated LF:HF ratio compared with Sham+PREB rats ($p = 0.053$, Fig. 2.4-2 e). After adjusting for multiple comparisons these changes were not statistically significant (Table 5). Other heart rate variability parameters were not different between groups (Table 5). The major finding was that

CIH exposure caused hypertension and cardiac autonomic imbalance, which were not alleviated by prebiotic supplementation.

2.4.4.5 Cardiorespiratory responses to 5-HT₃ receptor agonism evoking the cardiopulmonary reflex

Stimulation of 5-HT₃ receptors expressed on pulmonary vagal afferent nerve fibres, using PBG, evoked the integrated cardiopulmonary reflex. CIH exposure had no effect on hypotension, bradycardia, apnoea or post-apnoea induced tachypnoea associated with the pulmonary chemoreflex (Fig. 2.4-3 b-e). Prebiotic supplementation altered apnoea duration (Diet, $F(1, 41) = 4.950$, $p = 0.032$, $\eta^2 = 0.108$, Fig. 2.4-3b), however, *post hoc* analysis revealed no differences between groups. There was no significant difference between groups in all other parameters (Fig. 2.4-3c-e). The major finding was that pulmonary chemoreflex responses to vagal afferent stimulation were unaffected by CIH exposure.

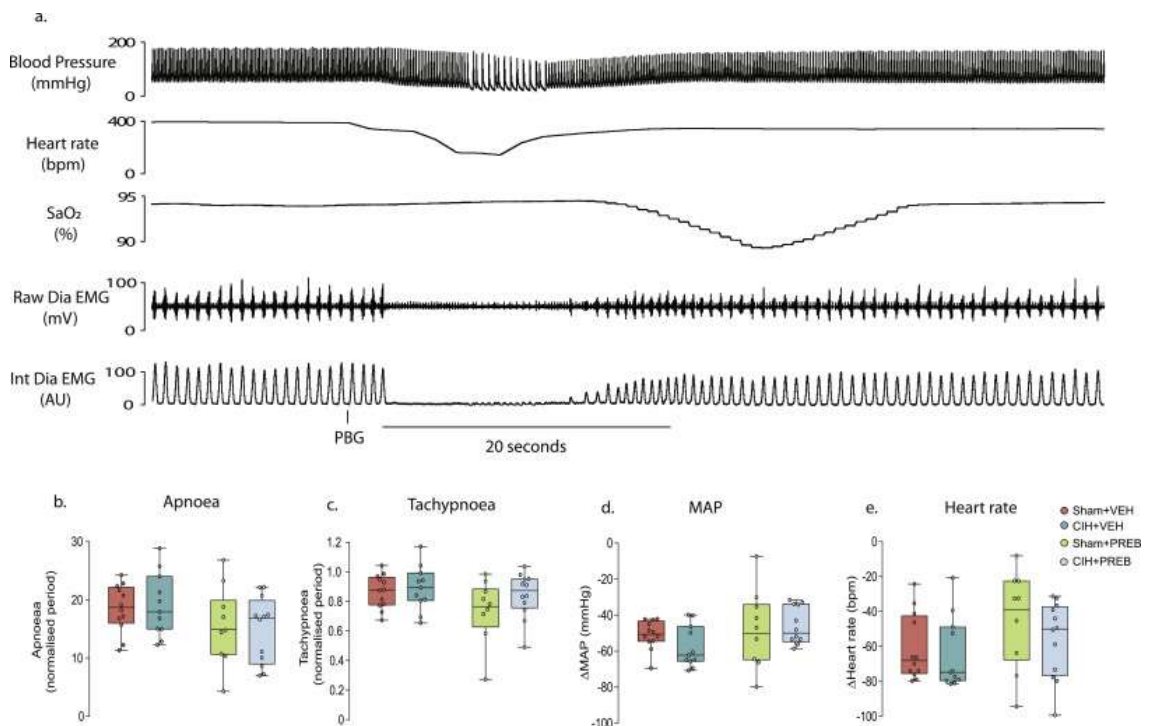


Fig. 2.4-3 a) Representative traces of blood pressure, heart rate, peripheral oxygen saturation (SpO₂) and raw and integrated diaphragm (Dia) electromyogram (EMG) activity during intravenous administration of the 5-HT₃ agonist, phenylbiguanide (25 $\mu\text{g}\cdot\text{kg}^{-1}$ i.v.). Group data for maximum apnoea duration (b) and tachypnoea (c)

normalised to respective baseline respiratory period in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB rats. Absolute change in MAP (d) and heart rate (e) in response to PBG in Sham, CIH, Sham+PREB and CIH+PREB rats. MAP, mean arterial blood pressure; CIH, chronic intermittent hypoxia; PREB, prebiotic; VEH, vehicle. Data (b-e) are expressed as box and whisker plots (median, IQR and minimum to maximum values); n = 9–12. Groups (b-e) were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test, where appropriate. CIH exposure had no effect on hypotension, bradycardia, apnoea or post-apnoea induced tachypnoea ($p > 0.05$; Fig. 2.4-3b-e). Prebiotic supplementation had a significant effect on apnoea duration ($p = 0.032$; Fig. 2.4-3b), with no effect on any of the other parameters ($p < 0.05$; Fig. 2.4-3c-e).

2.4.4.6 Cardiovascular responses to pharmacological blockade of sympathetic activity in anaesthetised rats

The blood pressure response to β_1 receptor antagonist administration (atenolol) was significantly increased by CIH exposure ($\chi^2(3) = 9.347$, $p = 0.025$). CIH+VEH was not different compared with Sham+VEH rats. There was a greater depressor response in CIH+PREB compared with Sham+PREB rats; the associated bradycardia was similar between all groups (Table 6). Intravenous infusion of the non-selective β -adrenoceptor blocker (propranolol), and sympathetic ganglion blocker (hexamethonium) evoked similar bradycardia and hypotensive responses across all groups (Table 6).

2.4.4.7 Pons and medulla oblongata neurochemistry

Comparison of L -DOPA and DOPAC concentrations in the pons, as well as DOPAC/DA, HVA, HVA/DA, 5-HT and 5-HIAA concentrations in the medulla oblongata revealed group differences ($p < 0.05$; Fig. 2.4- 4a, 2.4-4c-f, 2.4-4h, 2.4-4i). However, *post hoc* analysis revealed that monoamine, monoamine metabolites and precursors were not different in CIH+VEH compared with Sham+VEH rats in the pons or medulla oblongata (Fig. 2.4-4a-j). Pontine L -DOPA ($p = 0.020$) and medulla oblongata 5-HIAA ($p = 0.008$) concentrations were significantly increased, with medulla oblongata HVA ($p = 0.041$) levels decreased in CIH+PREB compared with CIH+VEH rats. CIH+PREB rats had increased pontine L -DOPA ($p = 0.038$) concentrations compared with Sham+PREB rats. Sham+PREB rats had reduced pontine DOPAC ($p = 0.021$) as well as medulla oblongata HVA ($p = 0.006$) and HVA/DA ($p = 0.016$) concentrations compared with Sham+VEH rats. Additionally, Sham+PREB rats had elevated pontine L -DOPA

($p=0.012$) concentrations compared with Sham+VEH rats. After adjusting for multiple comparisons, differences in pontine L-DOPA and medulla oblongata HVA concentrations in Sham+PREB compared with Sham+VEH and medulla oblongata 5-HIAA concentrations in CIH+PREB compared with Sham+PREB remained significantly different. In summary, prebiotic administration, but not CIH, altered brainstem neurochemistry.

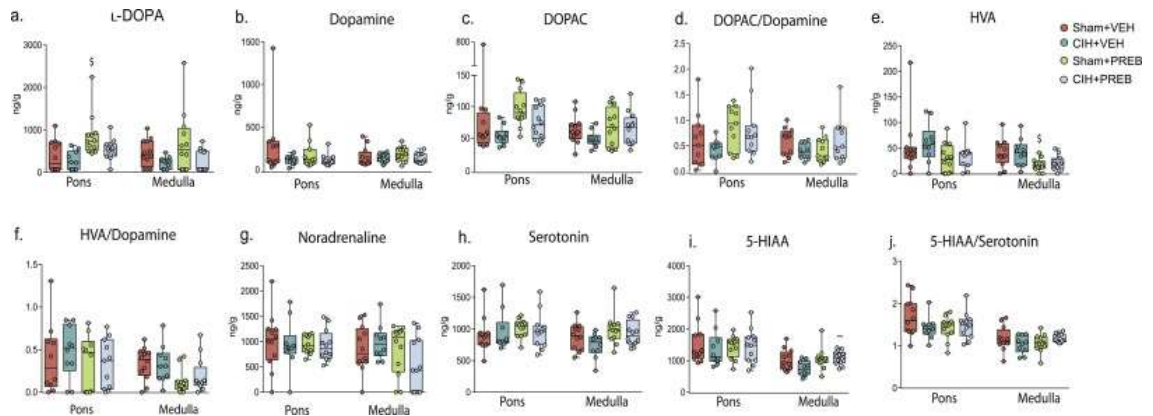


Fig. 2.4-4 Group data for L-DOPA (a), dopamine (b), DOPAC (c) DOPAC/Dopamine (d), homovanillic acid (e), homovanillic acid/dopamine ratio (f), noradrenaline (g), serotonin (h), 5-HIAA (i) and 5-HIAA/Serotonin ratio (j) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. L-DOPA, L-3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; CIH, chronic intermittent hypoxia; PREB; prebiotic; VEH, vehicle. Data (a-j) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 10-12$. Groups were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test, where appropriate. L-DOPA ($p = 0.003$; Fig. 2.4-4a) and DOPAC ($p = 0.006$; Fig. 2.4-4c) concentrations in the pontine region as well as DOPAC/DA (Diet*CIH, $p = 0.042$; Fig. 2.4-4d), HVA ($p = 0.001$; Fig. 2.4-4e), HVA/DA ($p = 0.020$; Fig. 2.4-4f), 5-HT (Diet, $p = 0.043$; Fig. 2.4-4h) and 5-HIAA ($p = 0.043$; Fig. 4i) concentrations in the medulla oblongata are different. Other monoamine, metabolites and precursors were not statistically different between groups ($p > 0.05$; Fig. 2.4-4b, 2.4-4g, 2.4-4h, 2.4-4j). ~ $p = 0.008$, CIH+PREB versus CIH+VEH; \$ $p = 0.006$, Sham+PREB versus Sham+VEH.

2.4.4.8 Plasma cytokine and corticosterone concentrations

Pro-inflammatory cytokines, IL-4 ($X^2(3) = 8.042$, $p=0.045$, Fig. 2.4-5c) and TNF- α ($X^2(3) = 10.784$, $p=0.013$, Fig. 2.4-5h) were different between groups. Post hoc analysis adjusted for multiple comparisons revealed that CIH exposure had no significant

effect on IL-4 or TNF- α levels. However, TNF- α and IL-4 were decreased in Sham+PREB compared with Sham+VEH rats. All other pro-inflammatory cytokines and corticosterone concentrations were not different between groups (Fig. 2.4-5a-b, 2.4-5d-g). In summary, CIH exposure had no effect on plasma cytokine or corticosterone concentrations. Prebiotic administration reduced TNF- α and IL-4 concentrations compared with VEH rats.

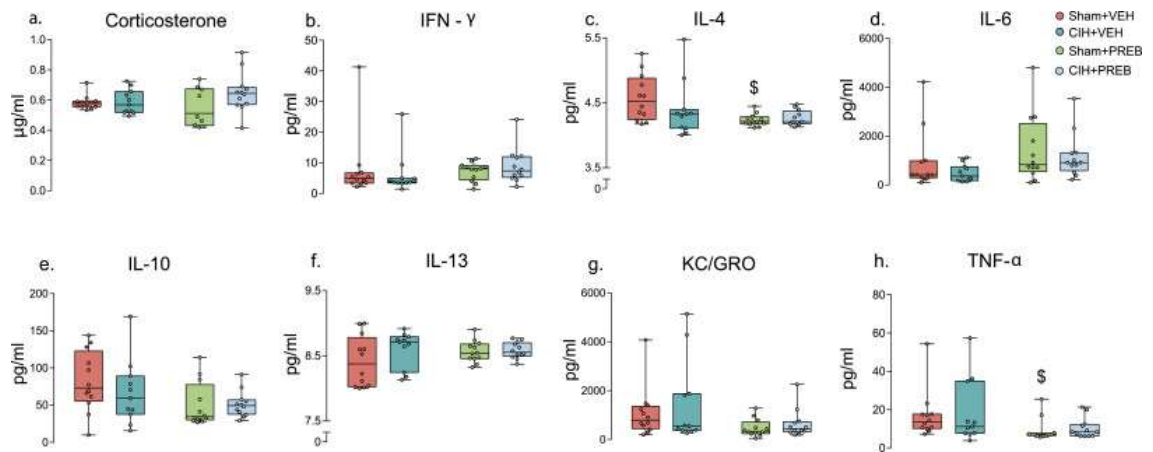


Fig. 2.4-5 Corticosterone and inflammatory mediators were equivalent between groups Group data for corticosterone concentration (a), IFN- γ (b), IL-4 (c), IL-6 (d), IL-10 (e), IL-13 (f), KC/GRO (g) and TNF- α (h) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. IFN- γ , interferon- γ ; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; IL-13, interleukin-13; TNF- α , tumour necrosis factor- α ; KC/GRO, keratinocyte chemoattractant/growth-related oncogene; CIH, chronic intermittent hypoxia; PREB, prebiotic; VEH, vehicle. Data (a-h) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 11-12$. Groups (a-h) were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test, where appropriate. Pro-inflammatory cytokines, IL-4 ($p = 0.045$; Fig. 5c) and TNF- α ($p = 0.013$; Fig. 5h) were affected by prebiotic administration. § $p = 0.003$, Sham+PREB versus Sham+VEH.

2.4.4.9 Caecal microbiota

2.4.4.9.1 Microbiota composition and diversity

CIH exposure had no effect on indices of alpha diversity (Fig. 2.4-6b-d). Prebiotic treatment significantly reduced bacteria species evenness in all statistical comparisons, indicated by decreases in Shannon and Simpson indices of alpha diversity (Fig. 2.4-6c-d). However, bacterial species richness, indicated by Chao1 index was not affected by prebiotic administration (Fig. 2.4-6b). These findings

suggest proliferation of a select number of bacterial species with no overall difference in bacterial richness. Principal component analysis revealed that CIH exposure did not affect β -diversity. Prebiotic administration shifted β -diversity in Sham+PREB and CIH+PREB compared with Sham+VEH and CIH+VEH rats, respectively (Fig. 2.4-6a, $p < 0.001$, PERMANOVA).

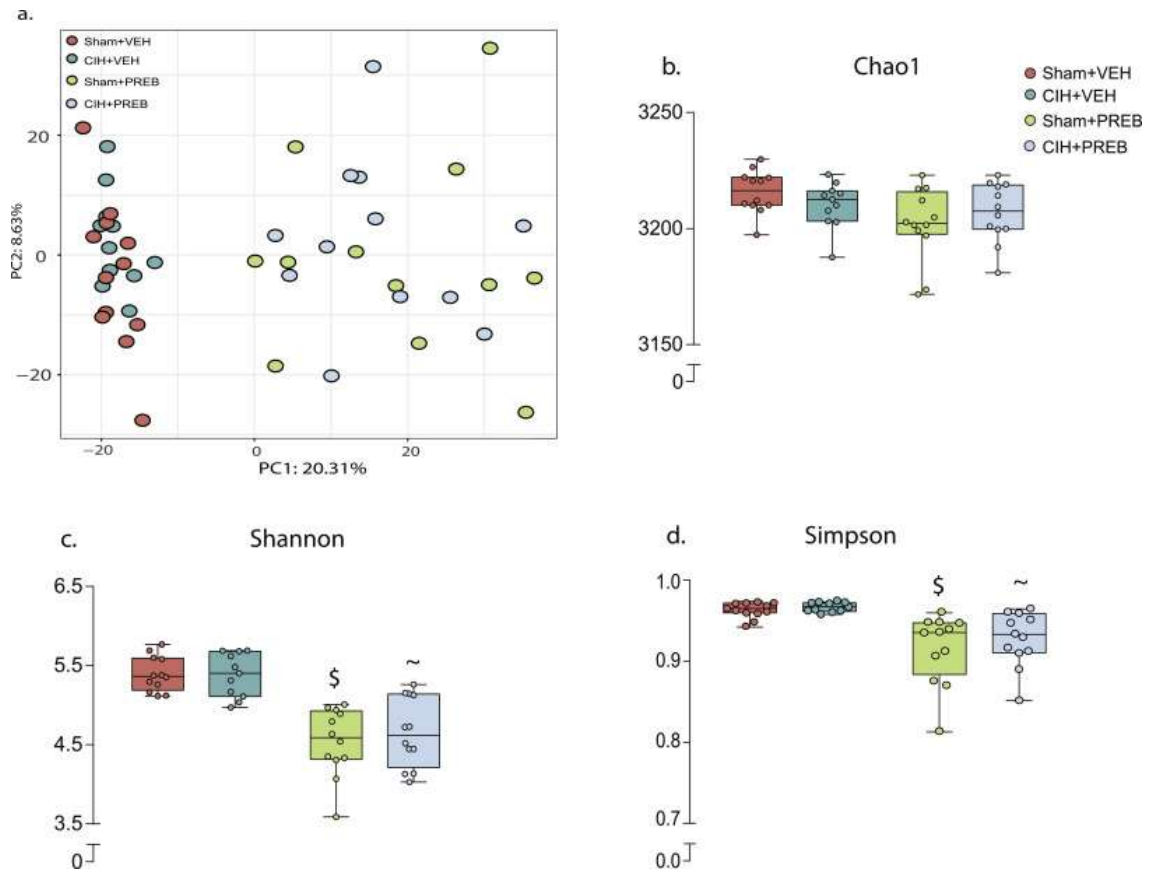


Fig. 2.4-6 Prebiotic administration alters rat caecal microbiota structure Group data for principal coordinate analysis (a) in 2-dimensional representations, Chao1 (b), Shannon (c), Simpson (d) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. CIH, chronic intermittent hypoxia; PREB; prebiotic; VEH, vehicle. Data (b-e) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 11-12$. Data (b-e) were statistically compared by non-parametric Mann-Whitney U test. P-value adjusted; ~ $p < 0.01$, CIH+PREB versus CIH+VEH; \$ $p < 0.0001$, Sham+PREB versus Sham+VEH;.

Whole-metagenome shotgun sequencing identified around 3200 bacteria species of 34 phyla. BH adjustment for multiple comparisons at bacterial species level did not reveal statistically significant differences between CIH+VEH and Sham+VEH rats given the exhaustive multiple comparisons performed. However, 3 large effect sizes (~ 1) were evident between CIH+VEH and Sham+VEH rats. Pathogenic species, namely, *Streptomyces sp. 452* and *Raoultella planticola* were increased and *Lactobacillus rhamnosus* (Fig. 2.4-6e), a beneficial commensal bacterial species, was decreased in CIH+VEH compared with Sham+VEH rats. Similarly, no statistically significant difference was evident between Sham+PREB and CIH+PREB rats, but large effect sizes (~ 0.8) were evident in 4 species. The pathogenic species *Helicobacter bilis* was increased whereas *Candidatus Gullanella endobia*, *Pectobacterium wasabiae* and *Corynebacterium striatum* were decreased in CIH+PREB compared with Sham+PREB rats (~ 0.8 effect size). A total of 420 bacterial species were statistically different in CIH+VEH rats compared with CIH+PREB rats, with 549 species different in Sham+VEH rats compared with Sham+PREB rats. The largest difference between these comparisons was due to a significant increase in the beneficial bacterial species *Bifidobacterium animalis* in the prebiotic groups.

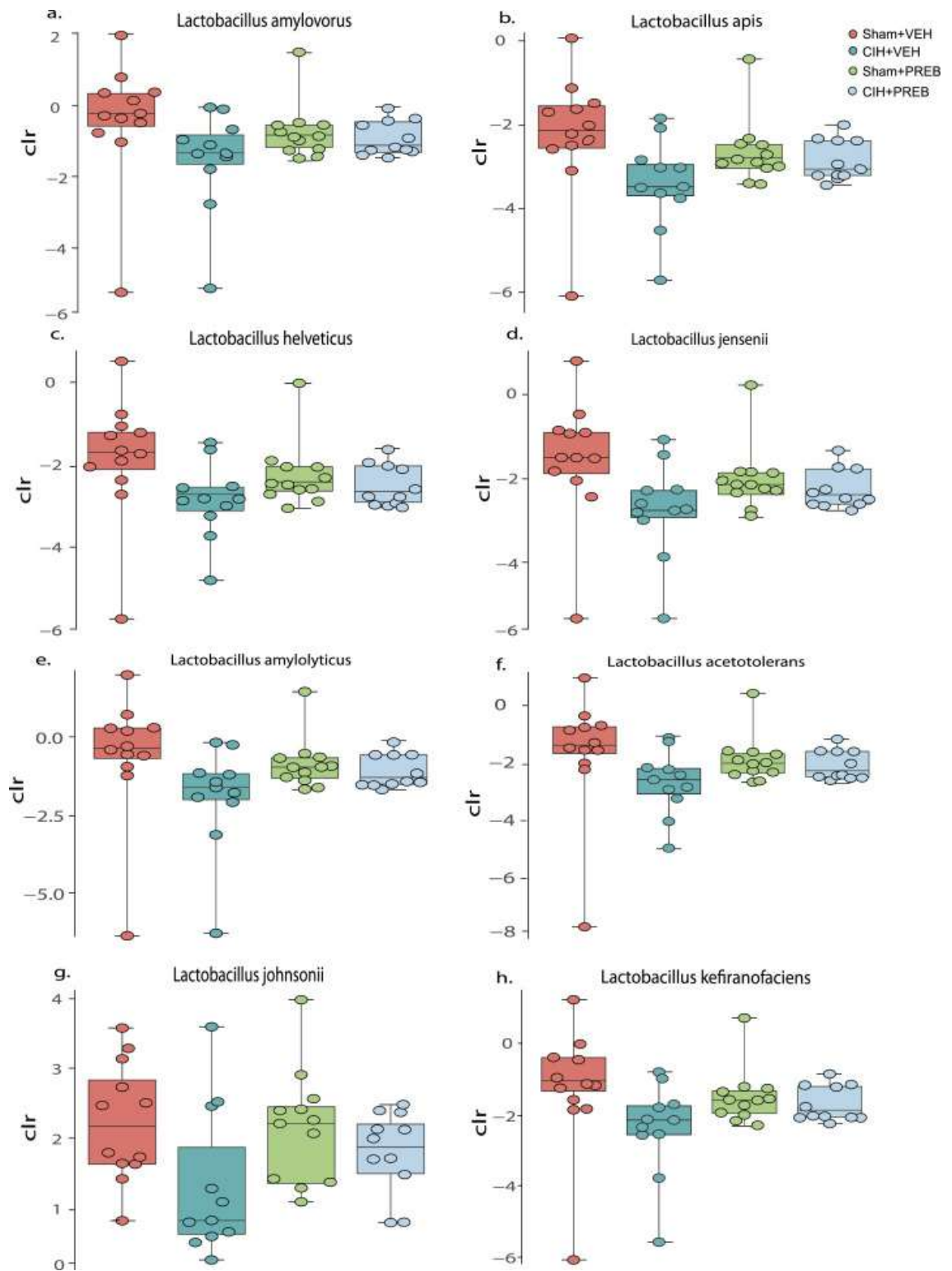


Fig. 2.4-7 *Lactobacilli* species are decreased in CIH+VEH compared with Sham+VEH
Group data for *Lactobacillus amylovorus* (a), *Lactobacillus apis* (b), *Lactobacillus helveticus* (c), *Lactobacillus jensenii* (d), *Lactobacillus amylolyticus* (e), *Lactobacillus acetotolerans* (f), *Lactobacillus johnsonii* (g) and *Lactobacillus kefirifaciens* (h) in

Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. CIH, chronic intermittent hypoxia; PREB; prebiotic; VEH, vehicle. Data (a-h) are expressed as box and whisker plots (median, IQR and minimum to maximum values); n = 11–12. Data (b-e) were statistically compared by non-parametric Mann-Whitney U test.

2.4.4.9.2 Gut-brain module and gut-metabolic module analysis

Using GBMs and GMMs we evaluated gut microbial functions. Our novel findings reveal that CIH exposure did not affect GBMs and GMMs analysis of caecal microbiota. Prebiotic administration altered the microbial potential of 10 and 31 GBMs and GMMs, respectively (adjusted $p < 0.05$). Several GMMs and GBMs were enriched (positive effect size) and reduced (negative effect size) in prebiotic treated rats compared with vehicle treated rats. Interestingly, GABA degradation ($p = 0.9$; effect size = 0.4, CIH+VEH *versus* Sham+VEH; $p = 0.9$, effect size = 0.4, CIH+PREB *versus* Sham+PREB; $p = 0.009$, effect size ~ 1 , CIH+PREB *versus* CIH+VEH; $p = 0.001$, effect size ~ 1 , Sham+PREB *versus* Sham+VEH; Fig. 2.4-7c) and butyrate synthesis I ($p = 0.9$; effect size = 0.4, CIH+VEH *versus* Sham+VEH; $p = 0.9$, effect size = 0.3, CIH+PREB *versus* Sham+PREB; $p = 0.2$, effect size = 0.6, CIH+PREB *versus* CIH+VEH; $p = 0.9$, effect size = 0.4, Sham+PREB *versus* Sham+VEH; Fig. 2.4-7d) abundance trended in diverging directions in CIH-exposed rats compared with Sham rats, depending on prebiotic or vehicle administration.

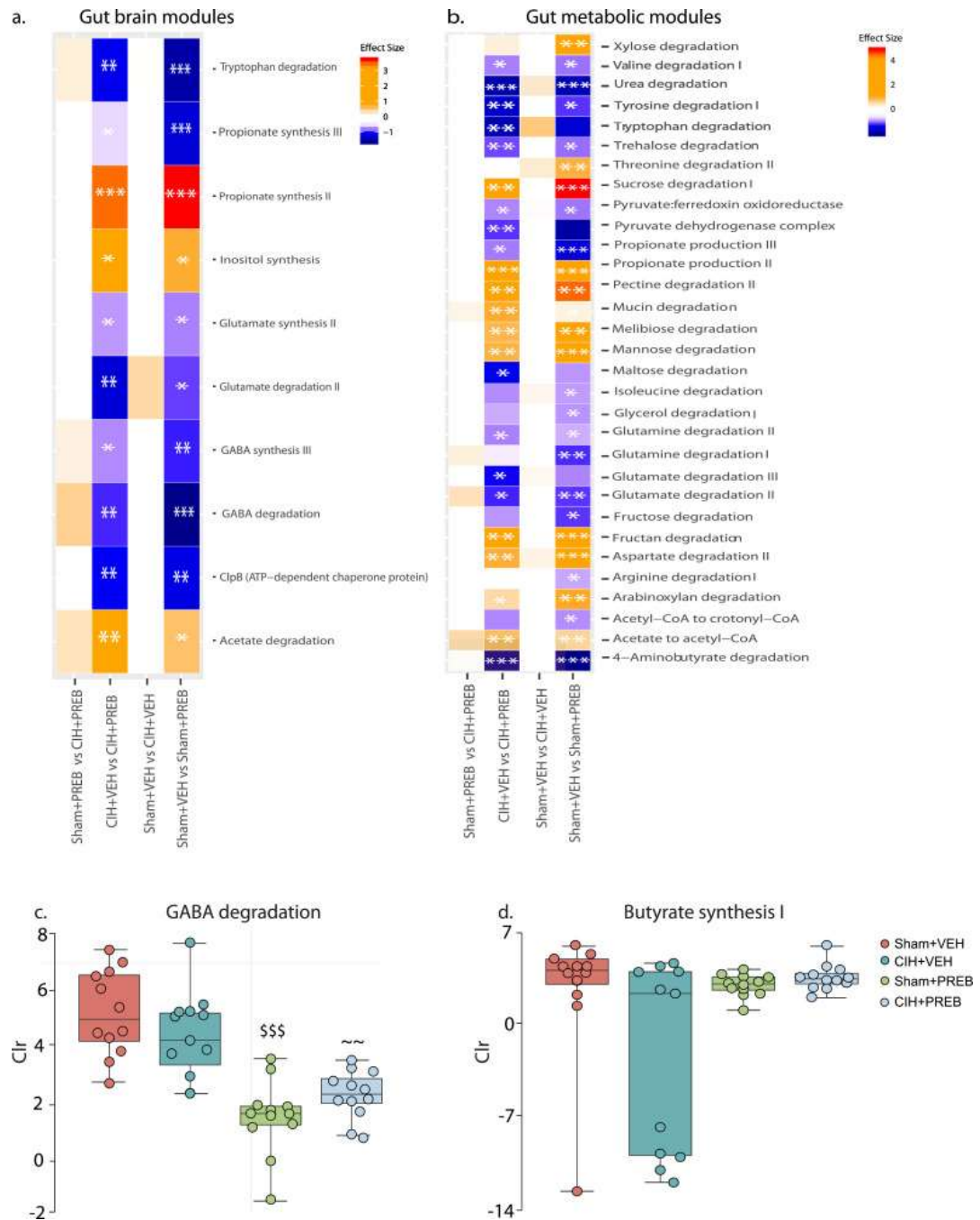


Fig. 2.4-8 Prebiotic administration alters GBMs and GMMs Group data for GBMs (a) and GMMs (b) in heatmap representation, GABA degradation (c) and Butyrate synthesis I (d) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. CIH, chronic intermittent hypoxia; PREB, prebiotic; VEH, vehicle. Data (c-d) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 11-12$. A pairwise implementation of the `aldex.ttest()` function was used to compare multiple groups. CIH exposure did not alter GBMs and GMMs. Prebiotic administration significantly modulated many metagenomes of the GBMs and GMMs. A positive effect size indicates an increase in prebiotic treated rats, a negative effect size

indicates a decrease in prebiotic treated rats. Benjamini-Hochberg corrected q -values, * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$. GABA degradation and Butyrate synthesis diverge in CIH-exposed compared with Sham rats, depending on prebiotic administration. ~ $p < 0.01$, CIH+PREB versus CIH+VEH; \$\$\$ $p < 0.001$, Sham+PREB versus Sham+VEH.

2.4.4.10 Correlation analysis

Using Hierarchical All-against-all correlation analysis the relative abundance of *Francisella sp. FSC1006* strongly negatively correlated with sigh frequency during hypercapnia in rats that did not receive prebiotics. No other significant correlations were evident. A total of 269, 16 and 110 bacterial species correlated with mean, diastolic and systolic blood pressure, respectively, when we independently investigated if blood pressure parameters correlated with bacterial species (Supplementary tables 9-11).

2.4.4.11 Faecal short-chain fatty acid concentrations

PCA analysis did not identify distinct clustering of CIH+VEH compared with Sham+VEH rats. However, separation of vehicle from prebiotic groups was evident (Fig. 8a). The loading plot (Fig. 8b) demonstrates this separation is due to higher concentrations of acetic, propanoic and hexanoic acid in prebiotic groups. Further analysis revealed that prebiotic supplementation significantly influenced faecal acetic ($X^2(3) = 22.420$, $p < 0.0005$, Fig. 8c) and propanoic ($X^2(3) = 11.211$, $p = 0.011$, Fig. 2.4-8d) concentrations. Prebiotic treatment significantly increased faecal acetic acid in all statistical comparisons (CIH+PREB versus CIH+VEH, $p = 0.002$; Sham+PREB versus Sham+VEH, $p = 0.001$; Fig. 2.4-8c), propanoic acid concentrations were increased in CIH+PREB compared with Sham+PREB rats ($p = 0.009$; Fig. 2.4-8d). There was no significant difference in other SCFA concentrations (Fig. 2.4-8e-h). CIH exposure had no effect on SCFA, whereas prebiotic administration increased SCFA concentrations in Sham and CIH-exposed rats.

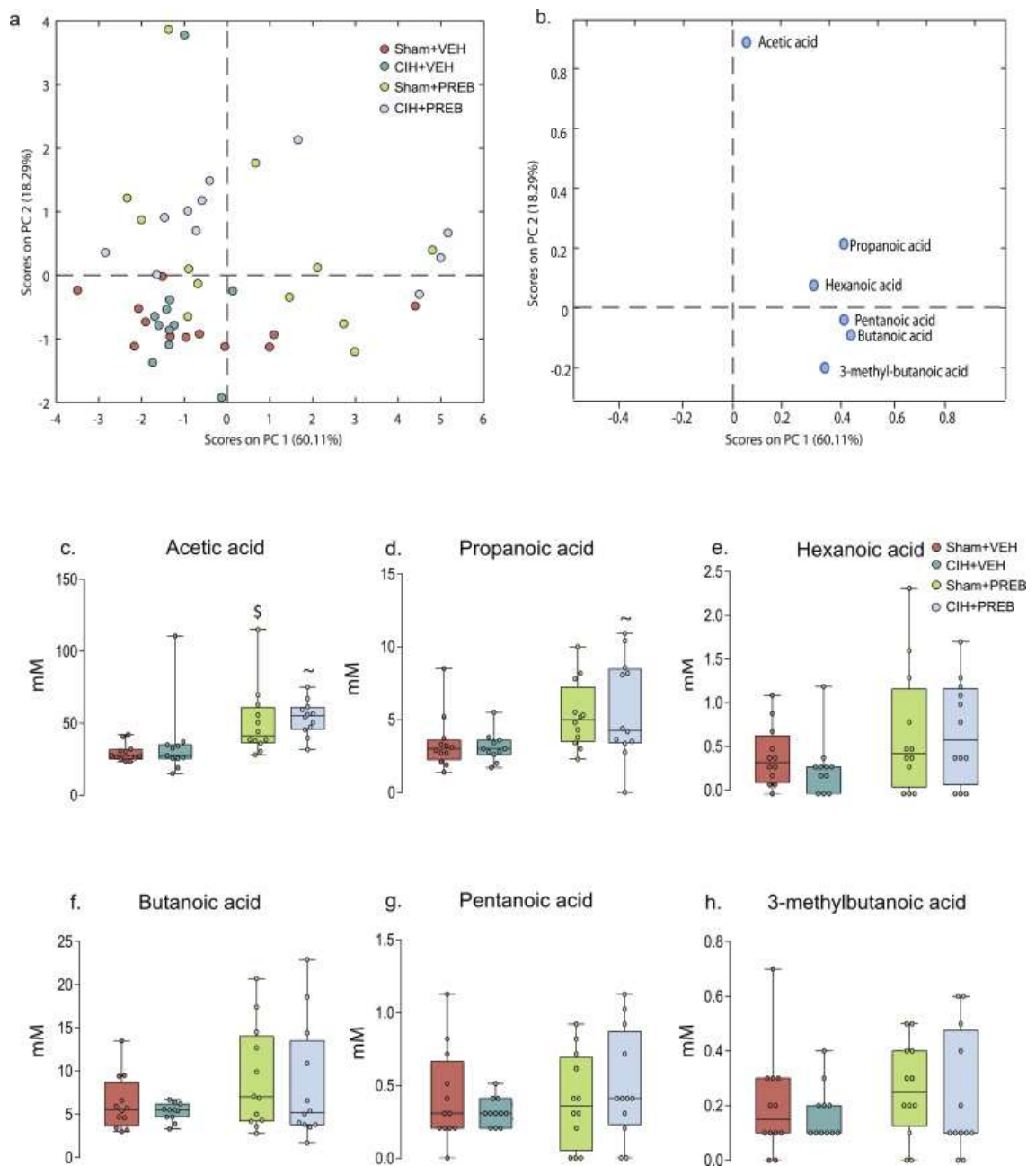


Fig. 2.4-9 Prebiotic administration increases faecal acetic and propanoic acid Score plot (a) from principal component analysis (PCA) model calculated on the relative concentrations of detected SCFA and loading plot (b) from PCA model calculated on the relative concentrations showing which variables are responsible for the pattern observed in (a). Group data for acetic acid (c), propanoic acid (d), hexanoic acid (e), butanoic acid (f), pentanoic acid (g) and 3-methylbutanoic acid (h) in Sham+VEH, CIH+VEH, Sham+PREB and CIH+PREB. CIH, chronic intermittent hypoxia; PREB; prebiotic; VEH, vehicle. Data (c-h) are expressed as box and whisker plots (median, IQR and minimum to maximum values); $n = 11-12$. Groups (c-h) were statistically compared using two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc where appropriate, or non-parametric Kruskal-Wallis test, followed by

Mann-Whitney U test, where appropriate. Acetic ($p < 0.0005$; Fig. 2.4-8c) and propanoic acid ($p = 0.011$; Fig. 2.4-8d) were increased as a result of prebiotic supplementation. All other SCFAs were not different ($p > 0.05$ Fig. 2.4-8e-h). ~ $p = 0.002$, CIH+VEH versus CIH+PREB; \$ $p = 0.001$, Sham+PREB versus Sham+VEH.

2.4.5 Discussion

There is a growing evidence-based consensus that the gut microbiota plays a modulatory role in physiological homeostasis. Recent studies posit that cardiorespiratory morbidity is linked to aberrant microbiota-gut-brain axis signalling (Ganesh et al., 2018; Meng et al., 2019; O'Connor et al., 2019; Toral et al., 2019). Investigations in rodents reveal that exposure to CIH disturbs the gut microbiota (Moreno-Indias et al., 2015; Moreno-Indias et al., 2016; Lucking et al., 2018). Exposure to CIH elicits cardiorespiratory dysfunction (O'Halloran, 2016; Laouafa et al., 2017; Elliot-Portal et al., 2018; Laouafa et al., 2019), predominantly considered to be mediated via CIH-induced carotid body sensitisation (Prabhakar et al., 2007; Iturriaga et al., 2009; Iturriaga et al., 2017), but also suggested to relate to aberrant signalling from other sites (Docio et al., 2018; Lucking et al., 2018). When viewed together, these observations encourage a new line of enquiry. Dysregulated microbiota-gut-brain axis signalling in CIH-exposed rodent models may play a modulatory role in cardiorespiratory disturbances evident in animal models of SDB. Manipulation of the gut microbiota via antibiotic administration/faecal microbiota transfer perturbs the gut microbiota and alters cardiorespiratory control (O'Connor et al., 2019). Prebiotic administration, promoting the expansion of beneficial microbes, could prove effective in the prevention of CIH-induced cardiorespiratory dysfunctions.

We sought to explore the interplay between cardiorespiratory physiology and the gut microbiota in a rat model of SDB, investigating if manipulation of the gut microbiota by prebiotic fibre administration could prevent or ameliorate cardiorespiratory dysfunctions evident in a CIH animal model. The principal novel findings of this study are: 1) CIH-exposed rats have reduced relative abundance of *L. rhamnosus*; prebiotic administration shifted the bacteria microbiota composition and diversity but did not restore *L. rhamnosus* relative abundance; 2) CIH exposure did not alter GBMs and GMMs analysis of caecal metagenomes; prebiotic administration modulated microbial functions; 3) CIH-exposed rats developed hypertension, which prebiotics failed to prevent; 4) CIH exposure had no effect on faecal SCFA concentrations; acetic and propanoic acid are increased in prebiotic groups; 5) CIH exposure increased the

apnoea index during normoxia, which was unaffected by prebiotic administration; 6) Monoamine, monoamine precursor and metabolite concentrations were unaffected by exposure to CIH; prebiotic administration had modest effects on brainstem neurochemistry; 7) Cardiorespiratory responsiveness to vagal afferent nerve stimulation was unaffected by CIH exposure; prebiotic administration had modest effects on apnoea duration; 8) CIH did not affect ventilation or metabolism; prebiotic administration increased ventilatory responsiveness to hypercapnia

Exposure to CIH elicited hypertension and a shift in autonomic balance towards sympathetic dominance, as evident by alterations in heart rate variability and spectral analysis parameters (Zoccal et al., 2007; Yamamoto et al., 2013). Furthermore, there was an elevated propensity for central apnoea apparent in CIH-exposed rats, (Julien et al., 2008; Edge et al., 2012; Donovan et al., 2014; Souza et al., 2015). There is considerable evidence supporting CIH-induced sensitisation of the carotid bodies, the principal peripheral oxygen sensors, with persistent elevation in chemo-afferent traffic to the NTS of the brainstem and resultant potentiation of sympathetic nervous outflow giving rise to hypertension (Fletcher et al., 1992; Kumar and Prabhakar, 2012; Iturriaga et al., 2015; Del Rio et al., 2016; Lucking et al., 2018). Carotid body ablation prevents CIH-induced hypertension and elevations in heart rate variability indicative of cardiac autonomic dysfunction (Iturriaga et al., 2015; Del Rio et al., 2016). Nevertheless, CIH-exposed guinea-pigs, with hypoxia-insensitive carotid bodies, have altered autonomic control of heart rate associated with modification in gut microbiota composition and diversity (Lucking et al., 2018). Moreover, exposure to severe CIH elicits sympathetic over-activity and hypertension in guinea-pigs in the absence of carotid body sensitisation (Docio et al., 2018), revealing sites beyond the carotid bodies that can contribute to the manifestation of CIH-induced hypertension.

Increased apnoea index, an observation commonly observed in CIH animal models, is proposed to manifest due to disturbances in the respiratory control network (Nsegbe et al., 2004; McKay and Feldman, 2008; Ramirez, 2014; Mateika et al., 2019). Carotid body plasticity and altered chemoreflex responsiveness is also suggested to be a driver of respiratory instability and apnoea (Prabhakar et al., 2007; Marcus et

al., 2010; Julien et al., 2011) and may have been a driver of apnoea in our model, although the lack of change in basal breathing and ventilatory responses to hypoxia in our study suggest a central origin. Numerous studies have recently linked the development of aberrant cardiorespiratory phenotypes, particularly hypertension, to perturbed gut microbiota, aberrant function profiles of gut microbes and altered SCFA production (Yang et al., 2015; Adnan et al., 2017; Li et al., 2017; Santisteban et al., 2017; Yan et al., 2017; O'Connor et al., 2019; Toral et al., 2019).

In our study, whole-metagenome shotgun sequencing revealed novel data showing that CIH+VEH hypertensive rats have modest alterations in bacterial species, however, gut microbial functional alterations were not different compared with Sham+VEH normotensive rats. The greatest bacterial species difference was in the relative abundance of the beneficial commensal bacterial species, *L. rhamnosus*, which was decreased in CIH+VEH compared with Sham+VEH rats. Interestingly, *L. rhamnosus* supplementation ameliorated CIH+HSD-induced hypertension in rats (Liu et al., 2019). Of note, the relative abundance of *L. rhamnosus* remained decreased in hypertensive CIH+PREB rats, suggesting that CIH-induced reductions in the relative abundance of *L. rhamnosus* may have contributed to the development of cardiovascular and autonomic dysfunction in our study, at least the development of hypertension. However, *L. rhamnosus* abundance was also reduced in Sham+PREB rats. If gut microbiota contribute to CIH-induced hypertension, it is more likely that a complex interplay of bacterial species contribute to the development of hypertension or the maintenance of normal blood pressure *per se*. In our study, a large number of bacterial species correlated with blood pressure parameters.

To our knowledge there are no studies using whole-genome shotgun sequencing to investigate the predicted gut microbial functions in OSA/hypertensive rodent models or animals treated with prebiotics. This area of research is in its infancy. In humans, numerous modules, essential for the host, were reduced and enriched in hypertensive compared with normotensive patients (Li et al., 2017; Yan et al., 2017; Kim et al., 2018). In our study, GBMs and GMMs were not altered in Sham+VEH compared with CIH+VEH rats and Sham+PREB compared with CIH+PREB rats, revealing that the predicted microbial function is unaltered as a result of CIH

exposure. Prebiotics had significant effects on gut microbial functions, resulting in increases and decreases in multiple modules important to the host. For example, increases in acetate degradation and propionate synthesis in prebiotic groups as predicted using GBMs and GMMs, coincides with elevated acetic and propionate acid, respectively in prebiotic treated rats.

Acetate-producing bacteria taxa, such as *Holdemania* were shown to be decreased in hypertensive rodents (Yang et al., 2015; Ganesh et al., 2018). Intriguingly, probiotic or prebiotic administration prevented the progression of hypertension in an OSA+HFD rat model, increasing caecal acetate and various SCFA producing bacteria that are diminished in the hypertensive rats (Ganesh et al., 2018). In our study, SCFA concentrations were unaffected by CIH exposure, yet, CIH-exposure caused hypertension, revealing that depletion of gut SCFAs is not obligatory for the development of CIH-induced hypertension. Prebiotic administration increased faecal acetic and propionic acid concentrations but had no beneficial effects on cardiovascular control; hypertension and enhanced heart rate variability were evident in CIH+PREB rats. In our study, elevations in SCFAs in prebiotic treated rodents may have had equal 'buffering effects' on blood pressure given that SCFAs act via olfactory receptor 78 (Olfr78) and G-protein-coupled receptor (Gpr41) to increase and decrease blood pressure, respectively having opposing effects on blood pressure regulation (Pluznick, 2013). This may contrast to outcomes in Ganesh *et al.*, 2018 wherein elevated acetate concentrations prevented the development of hypertension in OSA+HFD rodents, perhaps via Gpr41 receptor activation. Prebiotic administration did not ameliorate CIH-induced hypertension revealing that elevations in SCFA concentrations (or at least increases in acetic acid and propionic acid) or changes to microbial functional characteristics do not protect against CIH-induced hypertension.

We did not examine the effects of CIH exposure on intestinal function, but of interest CIH exposure did not increase plasma (or brainstem) pro-inflammatory cytokines in this study, although prebiotic administration decreased plasma TNF- α and IL-4 levels in Sham rats. Others have reported that CIH exposure in rodents increases plasma lipopolysaccharides (LPS) and elevates gut inflammation, contributing to intestinal

barrier dysfunction (Moreno-Indias et al., 2015; Wu et al., 2016), a phenotype also evident in other hypertensive models (Santisteban et al., 2017; Kim et al., 2018). Faecal microbiota transfer from donor normotensive to hypertensive recipient rats, and prebiotic and probiotic administration each independently decrease blood pressure, and prevent intestinal dysfunction and neuroinflammation in hypertensive models, including OSA animal models (Ganesh et al., 2018; Liu et al., 2019; Toral et al., 2019).

Prebiotic administration increased chemoreflex control of breathing in response to hypercapnia and hypoxic hypercapnia. Hypercapnia is primarily sensed by central chemoreceptors residing in the brainstem. Increased ventilatory responsiveness to hypercapnia is particularly interesting given that rats exposed to pre-natal stress exhibit altered ventilatory responsiveness to hypoxic and hypercapnic chemostimulation in adulthood, which correlated with changes in the gut microbiota (Golubeva et al., 2015). Moreover, antibiotic administration and faecal microbiota transfer were shown to perturb the gut microbiota composition and blunt chemoreflex control of breathing (O'Connor et al., 2019). The latter observation combined with findings from the present study suggests that the gut microbiota may shape brainstem responsiveness to carbon dioxide (acidosis) with implications for a range of respiratory control disorders.

We assessed monoamines and monoamine metabolites and precursors in the pons and medulla oblongata of the brainstem that are crucial in the neuromodulation of cardiorespiratory control. No significant modifications in monoamine, metabolite and precursor concentrations were evident in the brainstem of CIH-exposed rats. There was a trend for reduced dopamine turnover in the medulla oblongata of Sham+PREB rats, with elevated ventilatory responses to hypercapnia and hypoxic hypercapnia compared with Sham+VEH rats. This finding is particularly interesting given that antibiotic treated and faecal matter transfer rodents with perturbed gut microbiota each display blunted ventilatory responses to hypercapnia and exhibit increased brainstem dopamine turnover (O'Connor et al., 2019). Perhaps, D1 receptor activation may underpin elevated ventilatory responses to chemostimulation in Sham+PREB rats (Lalley, 2004). Previous studies demonstrate

that prebiotic administration affected DOPAC concentrations in the brainstem and frontal cortex of mice (Burokas et al., 2017). Yet, prebiotic administration did not alter other monoamine, metabolites and precursors of the dopaminergic pathway in animal models (Kannampalli et al., 2014; Burokas et al., 2017). CIH+PREB rats revealed significantly increased 5-HIAA concentrations in the medulla oblongata compared with CIH+VEH rats. Noteworthy, lesions of raphé serotonergic neurons and transgenic rodents without 5-HT neurones display reduced respiratory responsiveness to hypercapnic chemostimulation (Dias et al., 2007; Hodges et al., 2008; Li and Nattie, 2008; Hodges and Richerson, 2010). However, serotonin turnover was not different in CIH+PREB rats compared with CIH+VEH. It is not likely that serotonin mediated the elevated ventilatory drive to breathe in response to hypercapnia associated with prebiotic supplementation. Altered 5-HT receptor levels and 5-HT concentrations have been observed in the rodent pre-frontal cortex after prebiotic administration (Savignac et al., 2016; Burokas et al., 2017). Our data suggest that reduced dopamine turnover in Sham+PREB rats may be associated with increased ventilatory responses to hypercapnic chemostimulation.

The afferent vagal pathway is a pivotal signalling pathway of the microbiota-gut-brain axis, which responds to various stimuli including cytokines, bacterial metabolites including SCFAs, gut hormones and neurotransmitters (Goehler et al., 2000; Raybould et al., 2003; Johnston and Webster, 2009; Nohr et al., 2015). Central integration of vagal afferent signals occurs within the NTS of the brainstem. PBG, which is a 5-HT₃ receptor agonist, activates pulmonary vagal afferent C-fibres manifesting the pulmonary chemoreflex characterised by decreased blood pressure, bradycardia, apnoea and post apnoea-induced tachypnoea. Exposure to CIH did not affect the pulmonary chemoreflex but prebiotic administration increased apnoea duration. *Post hoc* analysis determined that there were no statistically significant differences between groups suggesting that vagal influence over these critical control centres was unaltered by any potential changes in microbiota-gut-brain signalling. Of interest, cardiorespiratory responses to PBG was also unaffected in other models of disrupted gut microbiota (O'Connor et al., 2019). Thus, notwithstanding alterations to the gut microbiota in CIH+VEH rats, as well as the notable changes in the gut

microbiota, SCFA concentrations, microbial functional characteristics and brainstem neurochemistry in prebiotic groups, no major differences in cardiorespiratory efferent responses to vagal afferent stimulation were observed revealing intact pulmonary chemoreflex circuit function in CIH-exposed and prebiotic supplemented animals. This does not preclude however, the possibility of altered vagal signalling from the gut in our models, which warrants attention in future studies.

2.4.6 Conclusion

Our novel findings add to the growing field investigating the role of the microbiota-gut-brain axis in the control of breathing and cardiovascular function (Ganesh et al., 2018; Liu et al., 2019; O'Connor et al., 2019). Herein we confirm that CIH exposure leads to the development of adverse cardiorespiratory and autonomic control, resulting in hypertension, cardiac autonomic imbalance and elevated propensity for apnoea. We revealed for the first time using whole-metagenome shotgun sequencing that a beneficial commensal species, *L. rhamnosus*, was decreased in CIH-exposed rats, but gut microbial functional characteristics were unaltered. Furthermore, faecal SCFA concentrations were not altered by CIH exposure. Prebiotic administration did not restore *L. rhamnosus* relative abundance in CIH-exposed rats and it decreased *L. rhamnosus* abundance in Sham+PREB rats. Prebiotics increased SCFAs and modulated GBMs and GMMs in CIH-exposed and Sham rats. Our findings suggest that microbiota-gut-brain axis signalling is unlikely to play a critical role in the development of the principal cardiovascular and respiratory maladies observed in CIH-exposed rats. These observations further support the pivotal role of the carotid bodies in the manifestation of CIH-induced cardiorespiratory malaise, which has relevance for human cardiorespiratory control disorders. Interestingly, ventilatory responses to hypercapnic and hypoxic hypercapnic chemostimulation were altered in prebiotic treated groups. Significant modulations to the gut microbiota may shape brainstem responsiveness to acidosis which has implications for homeostatic function of integrative body systems. Our findings extend previous knowledge of the relationship between the gut microbiota

and cardiorespiratory control in OSA animal models. Further studies are required to unravel discrepancies within the field, which will lead to a better understanding of the role of the microbiota-gut-brain axis in OSA animal models, and the potential use, where appropriate, of interventional adjunctive therapies focussed on the gut microbiota for the treatment of cardiorespiratory dysfunction.

2.5 Adolescent Dietary Manipulations Differentially Affect Gut Microbiota Composition and Amygdala Neuroimmune Gene Expression in Male Mice in Adulthood

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2.5.1 Abstract

Adolescence is a critical developmental period that is characterised by growth spurts and specific neurobiological, neuroimmune and behavioural changes. In tandem the gut microbiota, which is a key player in the regulation of health and disease, is shaped during this time period. Diet is one of the most important regulators of microbiota composition. Thus, we hypothesised that dietary disturbances of the microbiota during this critical time window may result in long-lasting changes in immunity, brain and behaviour. C57BL/6 male mice were exposed to either high fat diet or cafeteria diet, which consists of normal chow and a high caloric treat presented each day, during the adolescent period from postnatal (P) day 28 to P49 and were tested for anxiety and social-related behaviour in adulthood. Our results show long-lasting effects of dietary interventions during the adolescent period on microbiota composition and the expression of genes related to neuroinflammation or neurotransmission. Interestingly, changes in myelination-related gene expression in the prefrontal cortex following HFD exposure were also observed. However, these effects did not translate into overt behavioural changes in adulthood. Taken together, these data highlight the importance of diet-microbiota interactions during the adolescent period in shaping specific outputs of the microbiota-gut-brain axis in later life.

2.5.2 Introduction

Adolescence, the transition period from childhood to adulthood, is a critical developmental period characterised by the onset of puberty, as well as specific neurobiological processes and behavioural changes (Larsen and Luna, 2018). As adolescence is the last developmental stage before adulthood, it is not surprising that major refinement of neuronal structure and function during the adolescent period are associated with the maturation of social behaviours and cognitive abilities that promote individuation (Burnett et al., 2011). However, the growth and specialization of the adolescent brain that allow for maturation and adjustments to environmental challenges, also creates vulnerability and can result in maladaptation of brain circuits (Spear, 2013). Hence, adolescence is also a high-risk period for the onset of psychological disorders including psychosis, substance use disorders, eating disorders, impulse control disorders and affective disorders (Walker, 2002).

In recent years there is a growing appreciation that the microbiota, which describes the trillions of microbes including bacteria, archaea, eukarya, viruses and parasites living in and on their host, plays a key role in all aspects of host health including neurodevelopment and behaviour (Borre et al., 2014; Sampson and Mazmanian, 2015; Gilbert et al., 2018; Cryan et al., 2019). Hence, changes in the gut microbiota during the adolescent period are likely to impact brain and behaviour of the developing individual but are not well studied (McVey Neufeld et al., 2016; Cowan et al., 2019).

The microbiota during the developmental period is shaped by a variety of internal and external factors (Spor et al., 2011; Gilbert et al., 2018) which make the microbiota susceptible to external impact during development that might lead to maladaptation later in life. One of the most important factors that determines the composition of gut microbiota is diet. A corpus of studies have shown that different nutritional composition of the daily diet can drastically alter gut microbiota composition in humans and rodents (Klurfeld et al., 2018). These changes can occur rapidly and are transient in the adult rodents and humans. For example, consuming specific animal-based or plant-based diets alters the composition of the microbiota

composition within 24 hours and returns to baseline within 48 hours of termination of that specific diet (David et al., 2014; Singh et al., 2017) and microbiota composition of mice fed a high fat diet for 12 weeks reverts to baseline after 4 weeks (Zhang et al., 2012).

Regarding the microbiota-gut-brain axis there are a number of studies that show that dietary interventions in adulthood or early life can affect neurobiological, neuroimmune and behavioural changes (Sandhu et al., 2017). For example, exposure to high fat diet for either 18 days or 21 weeks results in impaired spatial learning in the Morris water maze (Denver et al., 2018) as well as in the object location memory and social memory during adolescence (Khazen et al., 2019; Yaseen et al., 2019), and increases levels of anxiety in the light/dark exploration test (Kang et al., 2014), as well as depressive-like behaviour in the trail suspension- and forced swim test (Vagena et al., 2019). Similarly, exposure to various forms of cafeteria diet, which is a variation of high fat and high sugar diet results in cognitive impairment in the novel object recognition task and the Morris water maze (Stranahan et al., 2008; Beilharz et al., 2014; Feijo et al., 2019) as well as alterations of anxiety-related behaviour in the elevated plus maze (Ferreira et al., 2018).

However, there is a paucity of studies focusing on the enduring effects of diet-induced changes in the adolescent microbiota-gut brain axis. We hypothesize that due to its relative instability, the adolescent microbiota is likely to be affected more drastically than the adult microbiota, which could result in long-lasting alterations in microbiota composition. To this end we investigate to which extent changing the diet from normal chow to either high fat diet (HFD) or cafeteria diet (CafD) during adolescence long-lastingly impacts microbiota composition and examine neurobiological, neuroimmune and behavioural outputs in adulthood.

2.5.3 Material and Methods

All experiments were conducted in accordance with the European Directive 2010/63/EEC, the requirements of the S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork (2012 #45) and the Health Products Regulatory Authority (HPRA). All efforts were made to reduce the number of animals used and minimise animal suffering.

2.5.3.1 Animals

36 male C57BL/6J0laHsd mice (Envigo, UK) were received at our facility at postnatal (P) day 21 and allowed to acclimatise for one week. Mice were marked and housed in groups of four in standard mouse cages. Mice were kept in ventilated rooms under a 12h light/dark cycle with lights on at 7.30am. Water and the diets described below were accessible *ad libitum*. The experimental timeline is depicted in figure 2.5-1.

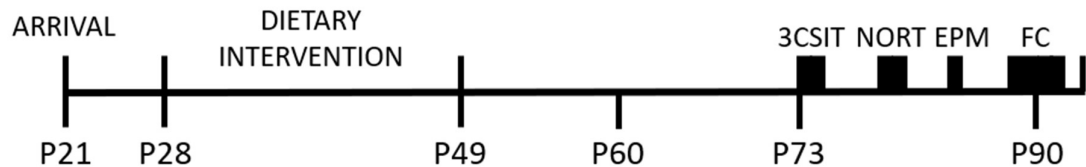


Figure 2.5-1: Experimental timeline. 3CSIT three-chamber social interaction task, EPM elevated plus maze, FC fear conditioning, NORT novel object recognition task.

2.5.3.2 Dietary Intervention

To impact gut microbiota during the adolescent period, three different diets were provided; standard diet, high fat diet (HFD) and cafeteria diet (CafD). Teklad Global 18% Protein Rodent Diet (2018S, Envigo, UK) was purchased as standard diet. This diet provided 3.1 kcal/g, 18% energy as fat, 58% as carbohydrate and 24% as protein.

HFD (D12451, Rodent Diet with 45% kcal% fat) was ordered from Research Diets Inc., USA. This diet provided 4.7kcal/g, 45% energy as fat, 35 % as carbohydrate and 20% as protein. Both diets were accessible *ad libitum*. CafD consisted of a variety of high caloric food items (Table 1) of which one was presented in addition to normal chow each day. To avoid strong individual differences in food intake of these items induced by hierarchy (Lee et al., 2018) all food items were presented in excess. Food items were weighed before presentation and the remainder was weighed after 24h. The information provided by the manufacturer was then used to calculate the energy intake (kcal). On average this diet provided 4.1 kcal/g, 36% energy as fat, 50% as carbohydrate and 14% as protein.

Animals were assigned to experimental groups (n=12) matching the average weight per cage (n=4), per treatment group (Ctrl 12 ± 1 g, 12 ± 1 g HFD, 12 ± 2 g CafD). At the onset of adolescence (P28) (Schneider, 2013), HFD or CafD were provided *ad libitum* for treatment groups. Control animals were provided with normal chow throughout the experiment. On P49 treatment groups were switched back to normal chow. Water was accessible *ad libitum* throughout the experiments. Bodyweight was measured in regular intervals to monitor weight gain.

Food Item	Company	Density kcal/g	Fat		Carbohydrate		Protein	
			per 100g		per 100g		per 100g	
			g	kcal	g	kcal	g	kcal
Oreo Cookie	Mondelez International	4.8	20.0	96.0	69.0	331.2	5	24.0
Craze milk chocolate Cereals	Aldi Own Brand	4.4	13.0	56.6	70.0	304.5	7.4	32.2
Mini Marshmallows	Aldi Own Brand	3.4	<0.5	<1.7	80.0	268.0	3.3	11.1

Doritos tangy cheese flavoured corn chips	Doritos	5.0	26.3	131.2	55.4	276.5	6.8	33.9
dried Banana	Tesco whole foods	3.0	1.1	3.3	67.1	199.3	4.5	13.4
Asian two minutes noodles	Nestle	4.4	18.3	81.1	57.8	256.1	9.9	43.9
Popcorn	Manhattan Peanuts	4.4	18.8	82.5	65.9	289.3	9.6	42.1
M&Ms peanut	Mars Chocolate France	5.1	25.3	129.3	59.2	302.5	9.7	49.6
roasted salted peanuts	Tesco Own Brand	6.2	50.5	312.1	10.6	65.51	27.2	168.1
Tapas crackers (leek&onion)	Valeo foods	5.1	25.0	126.8	60.0	304.2	8.7	44.1
Chocolate dairy milk	Cadbury	5.3	30.5	161.7	56.5	299.5	7.5	39.8
Kitkat	Nestle	5.1	24.8	126.7	27.2	139.0	2.4	12.3
Peanut butter	Tesco Own Brand	6.6	57.2	377.5	9.1	60.1	24.7	163.0

Table 1: List of food items presented during CafD treatment.

2.5.3.3 Behavioural Testing

Behavioural testing commenced three weeks after the end of treatment (P73). At least three days were allowed between tests for the animals to rest. Tests were performed between 9.00 am and 3.00 pm. Apart from the elevated plus maze, which was run under red light, all experiments were performed under dim light.

2.5.3.4 *Elevated Plus Maze*

The elevated plus maze (EPM) was used to assess anxiety-related behaviours. Anxiety-related behaviours have been shown to be influenced by the gut microbiota (Bravo et al., 2011; Savignac et al., 2014). The transfer of microbiota from an anxious strain to a less anxious strain for example causes increased anxiety in the recipient (Bercik et al., 2011). The EPM was carried out as described previously (Savignac et al., 2014). Mice were habituated to the testing room 60 min prior testing. The plus shaped maze was elevated 75 cm above the ground and consisted of two open and two enclosed arms (30 × 5 cm with 20 cm wall height). Mice were placed in the centre of the elevated plus maze facing the open arm and allowed to explore the maze for 5 min. The experiments were conducted under red light (5 lx). In between animals the maze was cleaned with 70% Ethanol. Experiments were recorded using a ceiling camera. The amount of entries into and time mice spent in the arms, as well as the time spent in the centre was manually scored by a blind observer. An entry into an arm was scored when all four paws entered.

2.5.3.5 *Three-Chamber Social Interaction Task*

It has been shown that social behaviour is dependent on the gut microbiota (Desbonnet et al., 2014; Stilling et al., 2015) and that the presence of microbiota during development is essential for the development of normal social behaviour (Stilling et al., 2018). Therefore, we assessed social behaviour in the three-chamber social interaction task. The test was conducted in a grey plastic box (36 x 19 x 30 cm, L x W x H) with three chambers interconnected by small openings as previously described (Stilling et al., 2018). A thin layer of fresh bedding was covering the ground and light levels were set to 60lx throughout the box. The test procedure consisted of three 10 min exploration trials, each starting with the mouse being placed in the middle chamber. During the initial phase, two empty wire cages (10 cm bottom diameter, 13 cm H) were placed in the outer chambers of the box and mice were allowed to explore for 10 min. In the second phase, an age-, sex- and strain-matched unfamiliar mouse and an inanimate object (rubber duck) were

placed in either of the wire cages and the test mouse allowed to explore for another 10 min. Following this session, mice were tested for social memory. They were placed back in the middle chamber and then allowed to explore a wire cage with the familiar mouse from the previous test session or a wire cage with an unfamiliar mouse. The time mice spent interacting with either of the wire cages was scored manually. Following, mice were moved back to their homecage.

2.5.3.6 Novel Object Recognition Task

Cognition is another parameter that has been shown to be impacted by microbiota composition (Davari et al., 2013; Desbonnet et al., 2015; Frohlich et al., 2016). We used the novel object recognition task to assess the memory in our animals. Novel object recognition is a well characterised test for working memory (Antunes and Biala, 2012) and was carried out essentially as described previously (Desbonnet et al., 2015). The arena was illuminated with 60lx. For this test the mice were habituated to a grey, plastic open field (40 cm x 30 cm x 25 cm, L x W x H) for 10 minutes. On the subsequent day, mice are placed in the same open field and exposed to two identical objects for 10 min. Following, mice were placed back in their homecage and allowed to rest for 1 hour. When they were placed in the open field one object had been replaced by a new object. Mice were again left to explore for 10min. The entire sessions were video-taped, and the time spent exploring either of the objects was scored manually by an observer blind to the experimental groups.

2.5.3.7 Differential Fear-Conditioning Paradigm

Hoban and colleagues had demonstrated that the gut microbiota is involved in amygdala dependent fear recall. Differential fear conditioning was adapted from Verma and colleagues (Verma et al., 2016) and run in a box with transparent front and metal side walls and evenly distributed metal rods as a floor (Med Associates, 30.5 cm x 24.1 cm x 21.0 cm). The chamber was illuminated with 80 lx. The box itself was enclosed by a sound-attenuating chamber. On day 1 of testing, mice were subjected to a differential fear-

conditioning paradigm in which an auditory stimulus (conditioned stimulus (CS+), 30 s white noise, 80 dB) was paired with a mild electric foot shock (unconditioned stimulus (US), 0.5mA for 2s). All mice received 5 CS+ with random inter-stimulus interval, starting 120 s after the mouse was placed in the box, followed by another 120 s without presentation of CS+/US at the end of conditioning. On day 2, mice were placed back in the same context as the previous day for 12 min to assess contextual fear. Between mice the box was cleaned with 70% Ethanol. For fear extinction and extinction recall on the following days, the grid floor was covered by white Plexiglas and a black triangle changing the dimensions of the box. Furthermore, the box was cleaned with 1% acetic acid instead of ethanol. For extinction, the CS+ was presented 25 times (30s, inter-stimulus interval 5 s) following a 120 s habituation period. Mice were removed from the box 120 s after the last CS+ presentation. For extinction recall on day 4, mice were placed in the same context but only presented with 5 CS+. Freezing was assessed by automated tracking software (Video Freeze[®], Med Associates Inc). The motion threshold index was set 18 and a minimum freezing duration of 30. Freezing during extinction was averaged into 5-trial blocks for analysis. Trial block 1 which consisted of the first 5 CS tones was used to depict memory retention.

2.5.3.8 Sample Collection

Mice were decapitated rapidly, and brain and gastrointestinal tract were removed. Randomly, 10 out of the 12 brains per group were gross dissected and the regions of interest snap frozen on dry ice. The other two were immersion perfused in 4% PFA and kept for analysis. Colon length, caecal weight and the amount of mesenteric fat were determined before samples were snap frozen. All samples were stored at -80 C until further processed.

2.5.3.9 Quantitative RT-qPCR

For quantitative RT-qPCR total RNA was extracted from the amygdala and the prefrontal cortex. Genes that were analysed were chosen based on having been shown to be influenced by the gut microbiota and/or their involvement in the behaviours under investigation. All genes analysed are listed in table 2. Total RNA was extracted with the mirVana total RNA extraction kit (Ambion, United Kingdom). RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) in a G-storm thermocycler (G-storm, Surrey, UK). Gene expression was analysed SYBR® Green real-time PCR was performed on the cDNA samples using SYBR green (SensiFAST™ SYBR®, BioLine) to evaluate gene expression levels. Gene expression levels were analysed on an AB7300 system (Applied Biosystems, Thermo Fisher Scientific). Expression levels were calculated as the average of three replicates for each biological sample from all three groups (n=8-9 per group) relative to β -actin expression. Fold changes were calculated using the $\Delta\Delta C_t$ method (van de Wouw et al., 2018).

2.5.3.10 Caecal content DNA extraction

The QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) was used for caecal DNA extraction. The procedure was coupled with an initial bead-beating step. Briefly, 200 mg of each caecal sample were homogenised in 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.0 mm sterile zirconia beads plus a single 3.5 mm diameter bead (BioSpec Products, Bartlesville, USA) with 1 ml of Qiagen InhibitEX® buffer as previously described (van de Wouw et al., 2018). Subsequent extraction steps followed the manufacturer's instructions. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) and the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Extracted DNA was stored at -20°C.

2.5.3.11 16S rRNA Gene Sequence-based microbiota analysis

Caecal microbiota collected at the end of the experiment (in adulthood) was used for 16S rRNA gene sequencing. No sequencing was done right at the end of the dietary intervention. Amplification and preparation for sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was done as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol and as previously described (Boehme et al., 2019). Briefly, 5 ng/μl of microbial genomic DNA was run with 1 μM of each primer (forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACH VGG GTATCTAATCC-3')) and 12.5 μl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK) in a total volume of 25 μl. PCR was run under the following conditions: initial denaturation at 95 °C x 3 min; 25 cycles of 95 °C x 30 s, 55 °C x 30 s, 72 °C x 30 s; and 72 °C x 5 min for final extension. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). Dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, USA). 5 μl of purified DNA with 5 μl index primer 1 (N7xx), 5 μl index primer 2 (S5xx), 25 μl 2x Kapa HiFi Hot Start Ready mix and 10 μl PCR grade water was amplified using the previous program with only 8 amplification cycles instead of 25. Thereafter, DNA was again purified using the Agencourt AMPure XP system. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Oregon, USA). Following, samples were run on the Agilent Bioanalyser for quality analysis and samples prepared for sequencing following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark), using a 2 × 300 cycle kit.

2.5.3.12 Microbiota Bioinformatics

Statistical analysis of microbiota data was performed in the R (version 3.6) software environment with Rstudio (version 1.1.453). Alpha diversity was calculated using the iNEXT library (Hsieh et al., 2016). Wilcoxon Rank Sum followed by Bonferroni post

hoc tests were used to assess difference in Alpha diversity scores. For principal component analysis (PCA), permutational multivariate analysis of variance (PERMANOVA) was used to identify relationships of significance between variables the *adonis()* function from the vegan library on Aitchison distance matrices calculated with the ALDEx2 library (Fernandes et al., 2013). A pairwise implementation of the ALDEx2 function *aldex.t.test()* was also used to calculate pairwise differential abundance. The benjamini-hochberg procedure was used to account for multiple comparisons, a q-value of 0.1 was deemed significant.

2.5.3.13 Statistical analysis

Data was checked for normality using D'Agostino & Pearson normality test. Behavioural results, physiological results and gene expression were analysed using a one-way ANOVA (post hoc Dunnett). Kruskal-Wallis test (post hoc Dunn) was used when data was not distributed normally. For body weight analysis as well as the learning curve and cued extinction in the fear conditioning paradigm two-way repeated measures ANOVA was used and corrected for multiple comparison using Tukey posthoc test. Significance was denoted with selection of a p-value of less than 0.05.

2.5.4 Results

2.5.4.1 Dietary intervention affects body weight

An overall effect of dietary intervention during the adolescent period on body weight was observed (repeated measures ANOVA for treatment $F(2, 33)=3.36$ $p=0.047$ and interaction $F(14,231)=2.195$ $p<0.001$, figure 2.5-2A). Tukey post hoc analysis showed that mice exposed to HFD had a significant increase of body weight at the end of treatment at day 21 ($p<0.01$), when compared to controls and displayed increased body weight compared to CafD treated mice from day 21 to day 51 ($p<0.05$). No difference in body weight was seen between groups at the end of the experiment on day

64.

2.5.4.2 Animals on HFD have higher energy intake during the treatment period

The energy provided by the different type of diets comes from different proportions of macronutrients available in the diet (figure 2.5-2B). While HFD provides the largest % of energy as fat, CafD like control diet provided the largest % of energy as carbohydrates. However, unlike control diet the % energy provided as proteins was much lower in CafD and the % energy provided as fat was elevated. Figure 2C depicts the chow intake of the three groups during the course of the experiment. An overall effect of dietary intervention during the adolescent period on chow intake was observed (repeated measures ANOVA for treatment $F(2, 6)=8.13$ $p=0.02$ and interaction $F(16, 48)=13.38$ $p<0.0001$, figure 2.5-2C). Tukey post hoc analysis revealed that mice exposed to CafD had a lower chow intake during the time of treatment in comparison to controls. This is an expected compensation for the increased intake of the food items in the cafeteria diet. There also is a significant increase in the amount of chow in HFD animals in the second half of the treatment period. This results in a different daily average energy intake per cage as depicted in figure 2.5-2D (Kruskal-Wallis test $H(2)=7.2$, $p=0.004$). Post hoc analysis

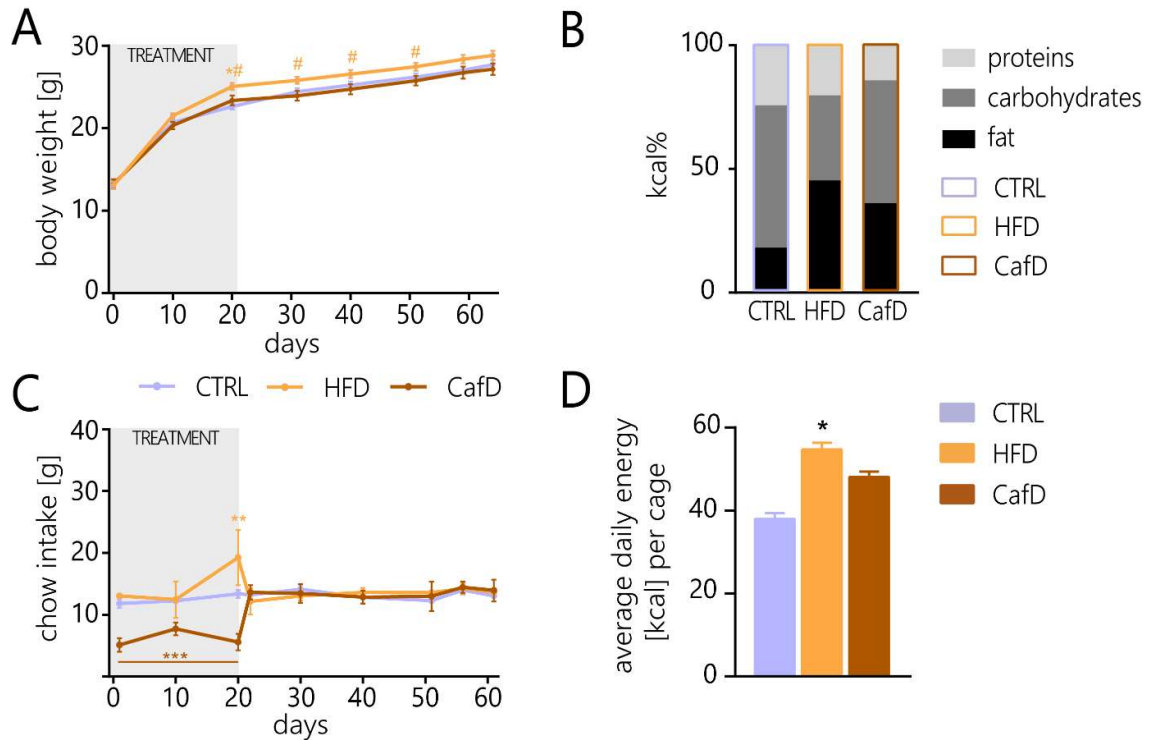


Figure 2.5-2 HFD but not CafD has enduring effects on Body weight. **A** Body weight was measured weekly during the experiment. Grey area highlights the period of dietary intervention. **B** Difference in kcal% of macronutrients depending on diet. The values for CafD represent the average macronutrient distribution over the entire treatment period. **C** Intake of chow and HFD during the experiment. Intake of treats of CafD are not included in this measure. **D** Average daily intake of energy per cage. Mean \pm SEM. **A, C & D** $n = 3$, **A & B** Repeated Analysis of Variance (ANOVA) post hoc Tuckey, **D** Kruskal Wallis post hoc Dunn: * significant difference between CTRL and treatment, # significant difference between CafD and HFD, *,# $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$

revealed that mice exposed to HFD show significantly increased energy intake per cage when compared to control mice and mice exposed to CafD ($p = 0.023$). When switched back to normal chow, no significant difference in chow intake can be observed between treatment groups (figure 2.5-2C).

2.5.4.3 Physiological readouts were not affected by adolescent dietary manipulations

Alterations in diet have been linked to changes in physiological measures of the gastrointestinal tract (Beraldi et al., 2015; Soares et al., 2015; Le Roy et al., 2019). Therefore, we measured caecum weight, colon length and the amount of visceral fat. No differences in either parameter were observed between mice that underwent dietary intervention during adolescence and control mice (caecum weight $F(2, 33)=0.677$ $p=0.515$, colon length $F(2, 33)=1.126$ $p=0.336$, visceral fat $F(2, 33)=0.623$ $p=0.543$, data not shown).

2.5.4.4 Enduring effects of dietary intervention on microbiota

To investigate the long-term effects of dietary intervention during adolescence on the gut microbiota, the caecal microbiota was analysed from adult mice at the end of the experiment. Long-lasting effects of dietary intervention on the microbiota composition and structure of adult mice were observed in mice exposed to either HFD or CafD during the adolescent period. HFD and CafD differentially affected alpha and beta diversity.

Alpha diversity, which describes the diversity of species in a community, was measured by calculating indices for richness and evenness based on ASV (amplicon sequence variant) level. Richness was estimated using Chao1. Figure 2.5-3A shows that there are no significant differences in the number of species within a group between control mice and mice that were exposed to dietary intervention during adolescence (pairwise comparison using Wilcoxon rank sum test, $p>0.05$). However, a significant decrease in Shannon diversity was observed in HFD treated mice (pairwise comparison using Wilcoxon rank sum test, followed by Bonferroni post hoc test, $p=0.011$), indicating a decrease in evenness across ASVs. No difference in evenness was seen for CafD treated mice ($p>0.05$).

On the beta diversity level, which describes the difference between communities, principal component analysis (PCA) identified long-lasting structural differences in microbiota following dietary intervention during adolescence (PERMANOVA followed by pairwise PERMANOVA; $p<0.001$ in all cases; figure 2.5-3B). Notably, the positive part of the second component is exclusively populated by subjects that were

exposed to dietary intervention (figure 2.5-3B blue area). The long-lasting effects of dietary intervention on the microbiota are also reflected by changes in the relative abundance of families (figure 2.5-3C). Members of the families *Ruminococcaceae*, *Lachnospiraceae*, *Erysipelotrichaceae*, *Coriobacteriaceae* and *Alcaligenaceae* are changed following both dietary interventions (figure 2.5-3C). However, differences in the structural properties of microbial communities between HFD and CafD treated mice compared to controls were observed in the relative abundance at the ASV level. Whereas a significant change in the relative abundance of *UCG-010* ($q < 0.05$), *NK4A214* ($q < 0.05$), one ASV of *Roseburia* ($q < 0.05$), 5 ASVs of the genus *NK4Q136* (all $q < 0.05$), two ASVs of *Lachnoclostridium* ($q < 0.05$), *Turicibacter* ($q < 0.05$), *Gordonibacter* ($q < 0.05$), two ASVs of *Enterorhabdus* ($q < 0.05$) and *Parasutterella* ($q < 0.05$) were observed in HFD-treated mice when compared to controls. Changes after CafD treatment only occurred in one ASV of *Roseburia* ($q < 0.05$), one ASV of *NK4A136* ($q < 0.05$), *Turicibacter* ($q < 0.05$) and one ASV of *Enterorhabdus* ($q < 0.05$) but showed a strong and significant increase in an ASV of *UCG-002* ($q < 0.001$). HFD and CafD treated mice differed significantly in the relative abundance of *Ruminiclostridium 9* ($q < 0.05$), *Anaerotruncus* ($q < 0.05$), *UCG-001* ($q < 0.05$), one ASV of *NK4A1136* ($q < 0.05$) and *Parasutterella* ($q < 0.05$). Overall, more genera are affected by HFD than CafD treatment but, genera seem to be more strongly affected by CafD than HFD. The ASV *UCG-002* is very strongly increased in CafD treated mice.

2.5.4.5 Altered Gene Expression in the Amygdala

During recent years, many studies have demonstrated a link between the gut microbiota and gene expression in the amygdala (Arentsen et al., 2015; Stilling et al., 2018). To understand to which extent short-term exposure to different diets and subsequent changes in the gut microbiota during the adolescent period could impact gene expression, we therefore focused on genes that have previously been shown to be impacted by the gut microbiota and to influence the behaviour under investigation.

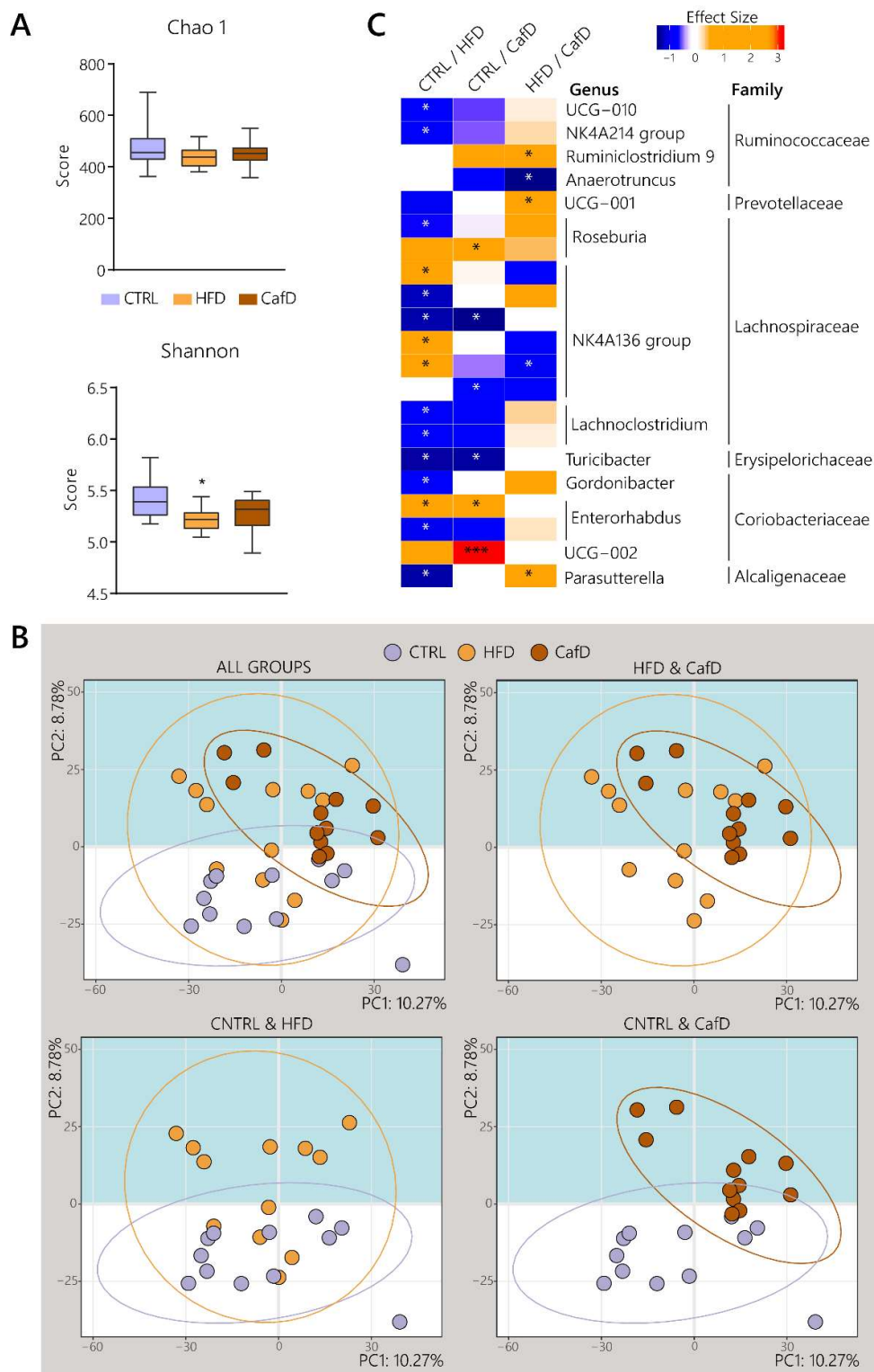


Figure 2.5-3 Long-lasting changes in Caecal Microbiota Composition following dietary intervention. **A.** α -diversity indices, Chao1 and Shannon Index depicting the richness and evenness of the sample, respectively, Mean \pm SEM. **B.** β -diversity, PCA blots of all groups together, HFD and CafD in comparison and HFD and CafD

compared to controls. **C.** Heat map representing relative abundance of ASVs that were significantly changed between controls, HFD and CafD mice. **A** Wilcoxon Rank Sum test, post hoc Bonferroni, * $p < 0.05$ **B** Permutational multivariate analysis of variance (PERMANOVA), followed by pairwise PERMANOVA post hoc Benjamini–Hochberg, **C** Mann–Whitney U test post hoc Benjamini–Hochberg, Benjamini–Hochberg false discovery rate (FDR) q value < 0.2 . Asterisks in the heat map represent the following q values: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.

We analysed various genes from whole amygdala lysates. 22 genes involved in neuroimmunity (Carrillo-Salinas et al., 2017), neurotransmission (Bravo et al., 2011; Hoban et al., 2018), tight junctions (Braniste et al., 2014; van de Wouw et al., 2018) or short chain fatty acid signalling (Erny et al., 2015; van de Wouw et al., 2018) were analysed. mRNA levels in the amygdala of adult mice were drastically changed following dietary intervention during adolescence in all clusters of genes analysed. Out of the 22 genes 19 were altered by adolescent exposure to HFD and 18 in CafD treated mice (figure 2.5-4F). Furthermore, genes were differentially affected by HFD or CafD treatment (results and statistics are summarised in Table 2). Of the genes involved in immune response, interleukin 1b (*Il1b*) was only increased in HFD, whereas interleukin 10 (*Il10*) was only increased in CafD treated mice. Gene expression for Fc fragment of IgG receptor IIb (*Fcgr2b*), toll-like receptor 4 gene (*Tlr4*), complement component 3 (*C3*), complement C1q A chain (*C1qa*), interleukin 8 receptor alpha (*Cx3cr1*) and Rho GTPase Rac2 (*Rac2*) was elevated following HFD and CafD treatment (figure 4A). This indicates that there are persistent changes in genes involved in neuroimmunity following adolescent treatment to diets.

Genes involved in neurotransmission were also upregulated (figure 2.5-4B). The gene expression of genes encoding for the gamma-aminobutyric acid type A receptor alpha2 subunit (*Gabra2*), dopamine receptor D1 (*Drd1*), metabotropic glutamate receptor type 5 (*Grm5*), corticotropin- releasing hormone receptor 1 (*Crhr1*), neuropeptide Y (*Npy*) and its Y1 receptor (*Npyr1*) was increased in mice following exposure to HFD and CafD during adolescence. However, gene expression for

glucocorticoid receptor NR3C1 was only increased in mice exposed to HFD and no change in gene expression was seen for the gamma-aminobutyric acid type B receptor subunit 1 (*Gabbr1*) for either HFD or CafD treated mice. Taken together these results suggest a high possibility for changes in neuronal transmission in the amygdala. Gene expression of genes associated with short chain fatty acids, free fatty acid receptor 1 (*Ffar1*) and the solute carrier subtypes (*Slc5a8* and *Slc16a1*) were also upregulated in both experimental groups (figure 2.5-4C). Gene expression for tight junction proteins, however, was affected differentially (figure 2.5-4D). Whereas gene expression for tight junction protein 1 (*Tjp1*) was upregulated in HFD treated mice, claudin 5 (*Cldn5*) was only upregulated in CafD treated mice. Occludin (*Ocln*) was upregulated in both experimental groups.

2.5.4.6 Altered gene expression related to myelination in the prefrontal cortex

The gut microbiota has been shown to be involved in the regulation of myelination in the prefrontal cortex which in turn influences anxiety (Gacias et al., 2016; Hoban et al., 2016; Lu et al., 2018). We therefore investigated whether myelination-related genes were affected in mice exposed to HFD or CafD during adolescence by analysing the gene expression levels of myelin regulatory factor (*Myrf*), the proteolipid protein 1 (*Plp1*) and the transcription factor sox-10 (*Sox10*) (figure 2.5-5). One-way ANOVA revealed that *Myrf* and *Sox10* were not changed by dietary intervention ($F(2, 25)=0.238$, $p=0.79$,

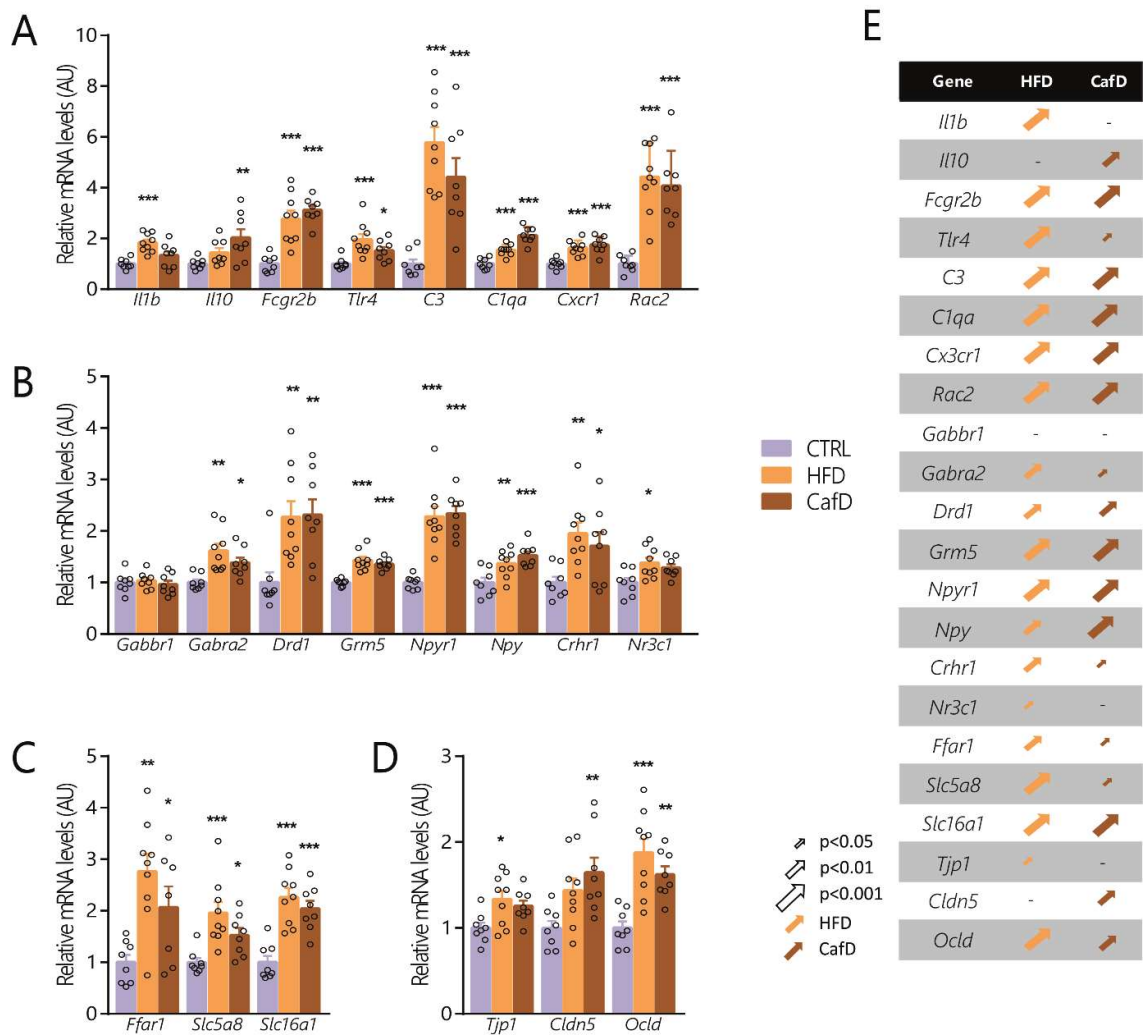


Figure 2.5-4 Altered Gene Expression in the Amygdala following dietary intervention. Gene expression related to **A** Neuroimmunity, **B** Neurotransmission, **C** Short chain fatty acids and **D** Tight junction proteins. **E** Overview of extent of changes in gene expression for all genes investigated. *C1qa* complement C1q subunit A, *C3* complement component 3, *Cldn5* claudin 5, *Crhr1* corticotropin-releasing hormone receptor 1, *Cx3cr1* interleukin 8 receptor alpha, *Drd1* dopamine receptor 1, *Fcgr2b* IgG receptor FcγRIIb gene, *Ffar1* free fatty acid receptor 1, *Gabbr1* gamma-aminobutyric acid type B receptor subunit 1, *Gabra2* gamma-aminobutyric acid type A receptor alpha2 subunit, *Grm5* glutamate metabotropic receptor type 5, *Il1b* interleukin 1b, *Il10* interleukin 10, *Npy*: neuropeptide Y, *Nr3c1* glucocorticoid receptor, *Ocld* occludin, *Rac2* Ras-related C3 botulinum toxin substrate 2, *Slc16a1* Solute Carrier Family 16 Member 1, *Slc5a8* Solute Carrier Family 5 Member 8, *Tlr4* toll-like receptor 4, *Tjp1* tight junction protein 1, *Npy1r* neuropeptide Y receptor Y1, Mean ± SEM. **A-D** n=8-9, **A** one way ANOVA post hoc Dunnett, except *Tlr4* Kruskal Wallis post hoc Dunn, **B** one way ANOVA post hoc Dunnett, except *Npy1r* Kruskal Wallis post hoc Dunn, **C** one way ANOVA post hoc Dunnett, **D** one way ANOVA post hoc Dunnett, * p<0.5, ** p<0.01, *** p<0.001

Sox1 $F(2, 27)=1.857$, $p=0.176$), whereas gene expression of *Plp1* was affected (*Plp1* $F(2, 27)=4.778$, $p=0.017$). Post-hoc analysis using Dunnett's multiple comparisons test revealed that *Plp1* was significantly elevated following HFD exposure during adolescence ($p=0.012$).

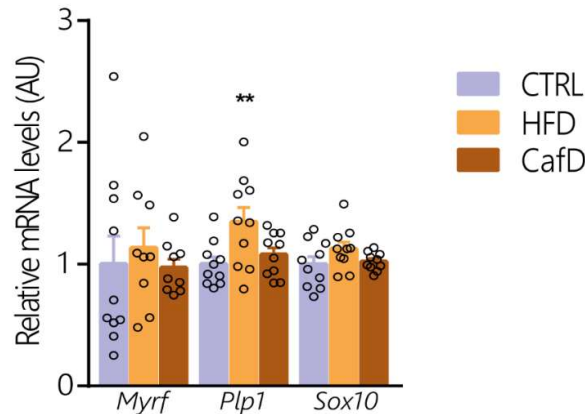


Figure 2.5-5 Changes in myelination related gene expression in the prefrontal cortex following HFD exposure. Genes related to myelination in the prefrontal cortex. *PLP1* is significantly upregulated following HFD exposure during adolescence. *Myrf* myelin regulatory factor; *Plp1* proteolipid protein; *Sox10* SRY-Box Transcription Factor 10. Mean \pm SEM, $n=9-10$, for all comparisons one-way ANOVA post hoc Dunnett, **, $p<0.01$.

2.5.4.7 No enduring behavioural effects of either dietary interventions during adolescence

To investigate possible long-lasting effects of dietary interventions and subsequent changes of microbiota composition during adolescence on behaviour, mice were run through a battery of tests in adulthood analysing aspects of anxiety, fear, sociability and memory. No differences in anxiety-related behaviour was observed in the elevated plus maze (figure 2.5-6A). Mice did not demonstrate differences in the % time spend in the open arm ($F(2, 27)=0.957$, $p=0.397$, figure 2.5-6A) or the % of entries made into the open arm ($F(2, 27)=3.36$, $p=0.05$, post-hoc analysis using Dunnett's multiple comparison test $p>0.05$ for both treatments, figure 2.5-6B). Similarly, social behaviour as measured in the three-chamber social interaction task (figure 2.5-6C) was not affected by dietary interventions. Mice did not show alterations in the time spent with either an inanimate object or a conspecific (2-way

ANOVA interaction $F(2, 54)=1.83$ $p=0.17$) or when exploring a familiar versus an unfamiliar mouse (2-way ANOVA interaction $F(2, 54)=1.774$, $p=0.179$ and treatment $F(2, 54)=0.854$, $p=0.432$, data not shown). This was further supported by the lack of differences in the total time the mice spent exploring both the inanimate object and the conspecific (Kruskal-Wallis test $H(2)=3.579$, $p=0.167$, figure 2.5-6D) or the familiar and the unfamiliar mouse ($F(2, 27)=0.097$, $p=0.908$, data not shown). When tested in the novel object recognition task dietary intervention has no effect on memory either (one-way ANOVA $F(2, 27)=0.187$, $p=0.831$, data not shown). Furthermore, dietary interventions during adolescence did not show any effects on fear conditioning. Mice show a normal learning curve (figure 2.5-6E). When exposed to a sequence of foot shocks mice showed no differences in the increase of % freezing over time (two-way repeated measures ANOVA $F(10, 135)=1.092$, $p=0.373$, and treatment $F(2, 27)=1.204$, $p=0.316$). Mice also did not show any differences in the % freezing when exposed to the training context (one-way ANOVA $F(2, 27)=1.294$, $p=0.291$ figure 2.5-6F), indicating no differences in hippocampal dependent fear learning. When placed in a different context and exposed to the tone, freezing response dropped similarly for all groups, hence, no difference was seen in cue extinction (two-way repeated measures ANOVA interaction $F(12, 162)=0.372$, $p=0.9712$ treatment $F(2, 27)=0.711$, $p=0.5$ figure 2.5-6F). Fear recall was tested thereafter and again dietary intervention did not have any effects on this parameter (one-way ANOVA $F(2, 27)=1.653$, $p=0.210$) (figure 2.5-6G).

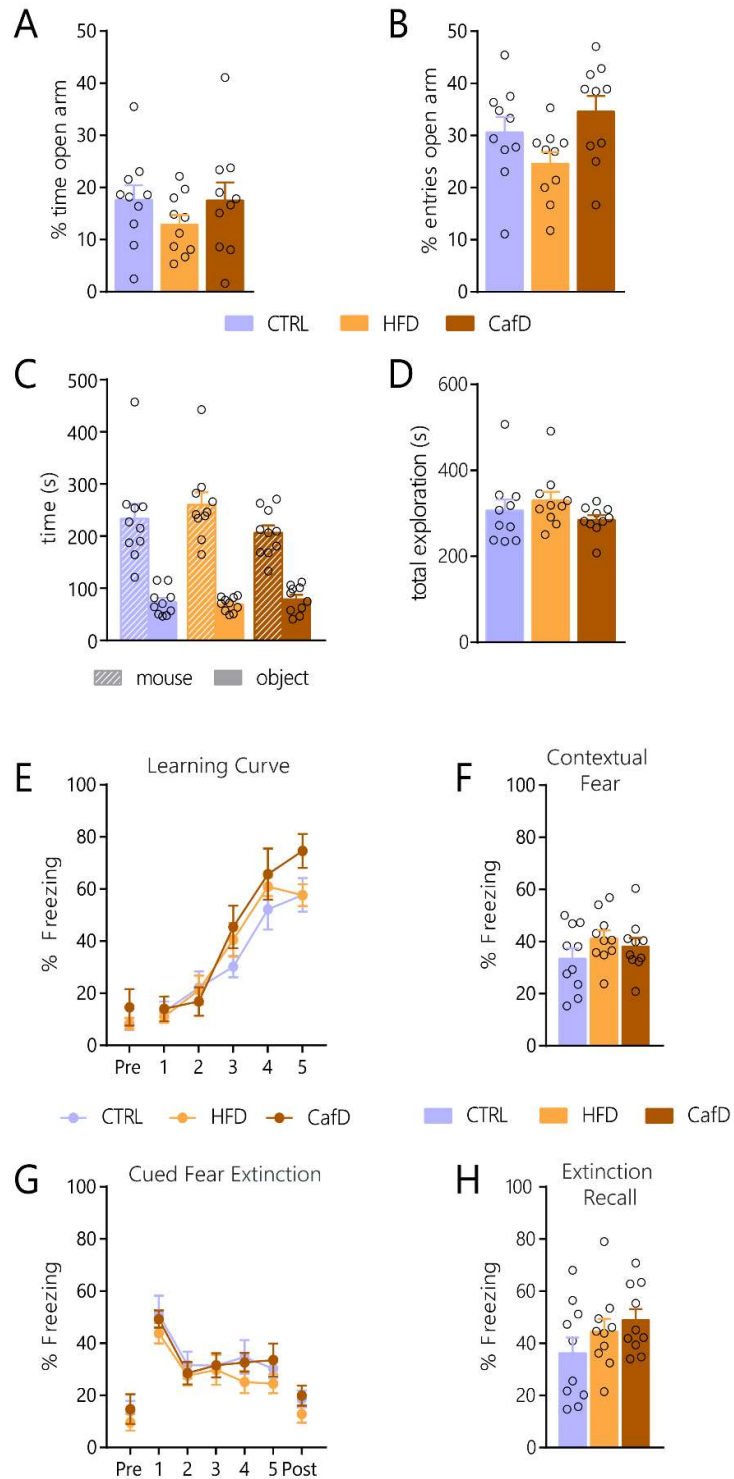


Figure 2.5-6 No effects of dietary intervention in adolescence on behaviour in adulthood. Behavioural parameters from the elevated plus maze **A**. % time spend in the open arm **B**. % entries into the open arm. **C**. Time spent interacting with the mouse and the object in the three-chamber social interaction task **D**. Total time of exploration for both object and mouse. Data from the fear conditioning paradigm **E**. Learning curve with the first foot-shock being administered after the pre-shock phase

*followed by 5 time bins following one foot-shock each. **F.** Total amount of freezing in contextual fear conditioning. **G.** Cued fear extinction **H.** Extinction recall. Mean \pm SEM, **A-H** n=10, **A & B**, one-way ANOVA post hoc Dunnett, **C** two-way ANOVA post hoc Bonferroni, **D** Kruskal Wallis post hoc Dunn, **E** two-way ANOVA post hoc Bonferroni, **F** one-way ANOVA post hoc Dunnett, **G** two-way ANOVA post hoc Bonferroni, **H** one-way ANOVA post hoc Dunnett.*

2.5.5 Discussion

In this study we assessed the long-term effects of short-term exposure to unbalanced diets during adolescence on gut microbiota composition as well as on molecular and behavioural parameters in adulthood. As gut microbiota composition is strongly influenced by dietary composition, we hypothesised that there would be differential enduring effects of such adolescent exposure to unbalanced diets and subsequent alterations to the adolescent gut microbiota on the adult microbiota composition as well as gene expression in the amygdala and the prefrontal cortex and behaviour. Interestingly, although we demonstrated that exposure to three weeks of HFD or CafD during the critical adolescent period results in significantly altered microbiota composition and altered gene expression in adult mice these changes did not translate to enduring alterations in overt changes in the behaviours under investigation. Thus, our hypothesis was only partially confirmed.

In these studies we show a persistent effect of diet on the microbiota which are in line with previous studies that have demonstrated effects of a variety of deleterious diets on human and rodent gut microbiota (Brown et al., 2012; David et al., 2014) and it is known that different dietary components differentially shape microbiota composition (Albenberg and Wu, 2014; Klurfeld et al., 2018). For example, exposure to cafeteria diet has caused alterations in the abundance of the phyla Firmicutes, Actinobacteria and Proteobacteria (Del Bas et al., 2018) in rats, whereas long-term treatment with high fat diets has altered Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria (Kim et al., 2012; Daniel et al., 2014; Kim et al., 2019). Obviously, depending on the composition of high fat and control diet (Huang et al., 2013), length of treatment (Kim et al., 2019) and age period (Villamil et al., 2018), changes on the phylum, family or genus level can differ. For example, 8 weeks treatment of C57BL/6J mice with high fat diet (60 kcal % fat, Research Diets) resulted in increase of *Ruminococcaceae* and *Rikenellaceae* (Kim et al., 2012), whereas a 12 weeks treatment of C57BL/6NCrl mice with high fat diet (60 kcal % fat, Ssniff GmbH) resulted in a decrease of *Ruminococcaceae* and an increase of *Rikenellaceae* (Daniel et al., 2014). However, overall *Ruminococcaceae*, *Rikenellaceae* and *Lachnospiraceae* seem to be the families that are commonly affected by diet with high fat content

(Kim et al., 2012; Daniel et al., 2014; Lecomte et al., 2015; Reichelt et al., 2018). This is supported by a recent meta-analysis study of the effects of high fat diet on the microbiota in mice stating that *Ruminococcaceae* and *Lachnospiraceae* are highly enriched following high fat diet treatment (Bisanz et al., 2019). Such changes in microbiota composition agree with those observed in the current study with changes in the phyla Firmicutes and Actinobacteria following CafD exposure and altered abundances of Firmicutes, Proteobacteria but also Actinobacteria in adulthood observed following HFD treatment during adolescence. Furthermore, we observe changes in *Ruminococcaceae* and *Lachnospiraceae* in HFD treated mice and changes in *Lachnospiraceae* in CafD treated mice.

The different effects of HFD and CafD on phylum, family and genus level observed in this study are most likely induced by differences in diet composition and consumption. Mice exposed to HFD consumed more fat and less carbohydrates than control mice, whereas mice on CafD consumed more fat and a similar level of carbohydrates but consumed far less proteins than the control animals. Furthermore, it is worth noting that as fat in the CafD was provided by a variety of different food items in addition to the normal chow, lipids and fatty acids provided by this diet differed in source, type and quantity from the fat provided by HFD. Thereby, the dietary composition and the consumption are likely to have differentially impacted microbiota composition.

The effects of dietary intervention seen on genus level, in comparison to what is observed in the literature might be not only due to differences in dietary composition but also the difference in treatment time. It is worth noting that in most common dietary intervention studies mice are exposed to the diet for long periods without interruption and are usually still receiving the diet at the termination of the experiment lab (Boitard et al., 2014; Boitard et al., 2015; Labouesse et al., 2017; Hassan et al., 2018; Reichelt et al., 2018), whereas mice in our study only had the diet during a critical developmental time period - adolescence. The fact that the effects of these diets on the microbiota and brain can still be observed even after 47 days of normal chow exposure, could be explained by the developmental instability of the

gut microbiota during adolescence. Microbiota composition in adulthood is considered rather stable, however, it only acquires its stability by slowly being shaped by internal and external factors during the developmental period (Walsh et al., 2014). Therefore, changes to the microbiota composition during the adolescent period are likely to have a far bigger and longer-lasting impact than when exposed to the same external factor in adulthood (Borre et al., 2014). Hence, our data suggests, that the impact of dietary intervention on the gut microbiota during adolescence is long-lasting, and that microbiota composition does not recover fully after exposure to either HFD or CafD. However, the data available on the regeneration of the gut microbiota following exposure to unbalanced diet in adulthood is inconsistent. Whereas exposure to four weeks of 60% high fat diet results in an altered microbiota composition even after 21 weeks of normal chow (Thaiss et al., 2016) switching back to normal chow after a 8 week exposure to 60% high fat diet was sufficient to normalise changes observed in gut microbiota composition (Safari et al., 2019). Overall, these data strongly suggest that gut microbiota composition was changed during the adolescent period and did not recover fully from this exposure until the end of the experiment. However, further studies are therefore needed to investigate whether indeed the same treatment in adulthood would result in changes in gut microbiota composition after switching to normal chow.

Diet induced changes in gut microbiota have been linked to alterations in immune response (Zinocker and Lindseth, 2018), neurotransmission (Hassan et al., 2018), and alterations of the microbiota by other factors has been implicated in myelination (Gacias et al., 2016; Hoban et al., 2016). As we were interested in the effect of changes in the microbiota during the adolescent period, we focused on gene expression analysis in the amygdala and the prefrontal cortex, as it has been shown previously that gene expression in these areas is strongly affected by microbiota during development (Stilling et al., 2015; Hoban et al., 2016). Here we show that dietary intervention during the adolescent period leads to permanent changes in gene expression of genes involved in neurotransmission, short chain fatty acid transport and signalling

or blood brain barrier integrity in the amygdala, as well as genes involved in myelination (Plp1) in the prefrontal cortex in HFD treated mice.

The microbiota, not only during the developmental period but also thereafter plays an important role in the induction and training of the host immune system and the symbiotic relationship with the microbiota is essential for proper immune system functioning (Belkaid and Hand, 2014). Changes in microbiota composition induced by dietary intervention therefore are likely to impact the immune system. Furthermore, excessive consumption of diets rich in fat results in increased secretion of pro-inflammatory cytokines (Thaler et al., 2012) and disruption of blood-brain-barrier integrity (Kanoski et al., 2010; Guillemot-Legris and Muccioli, 2017; Reichelt et al., 2018). Gene expression for the pro-inflammatory cytokines Il1b was upregulated in mice exposed to HFD and the anti-inflammatory cytokine Il10 was upregulated in CafD treated mice, indicating that there is a possibility for differential immune response activation in adulthood following dietary intervention during adolescence. Other genes involved in immune response, however, were elevated in both dietary intervention groups. These include genes involved in microglial-mediated phagocytosis and synaptic remodelling as well as components of the innate immune systems (Fcgr2b, Rac2, C3, C1qa, Cx3cr1, Tlr4), indicating that overall immune system activity is elevated resulting in neuroinflammation. Increased levels of neuroinflammation would suggest a decrease in blood-brain-barrier integrity, which plays an important role in regulating the brain's response to inflammation (Zlokovic, 2008). We find elevated levels of expression of genes involved in blood-brain barrier integrity which are differentially affected by dietary intervention. CafD seems to be affecting blood barrier-related genes more drastically than HFD as gene expression of claudin 5, which is thought to be the dominant tight junction protein, is significantly increased in CafD but not in HFD treated mice. HFD treatment on the other hand increased gene expression of tight junction protein ZO-1 and occludin, which could still

translate into changes of blood-barrier permeability as it was shown that these proteins play a much bigger role in blood-brain-barrier integrity than previously assumed (Berndt et al., 2019). Interestingly, blood-brain-barrier integrity is decreased in germ-free mice and rescued upon colonisation of germ-free mice, indicating that an increase of blood-brain-barrier integrity is possible following changes in gut microbiota composition (Braniste et al., 2014).

The gut microbiota produces short-chain-fatty acids by fermentation of indigestible polysaccharides. These have been implicated in gastrointestinal homeostasis, immune regulation, host metabolism but also in brain and behaviour (van de Wouw et al., 2018; Li et al., 2019; Vagena et al., 2019). Dietary intervention has been shown to affect short-chain-fatty acid metabolism (Maciejewska et al., 2018), which in turn can affect brain homeostasis and behaviour (van de Wouw et al., 2018; Vagena et al., 2019). Here we observe changes in the expression of *Ffar1* and *Slc5a8* and *Slc16a1* indicating that transportation and signalling of short-chain fatty acids following dietary intervention in adolescence have changed. Furthermore, classical and peptidergic neurotransmission is likely to be affected by dietary intervention during the adolescence period, as gene expression is changed for the genes encoding the GABA receptor $\alpha 2$ (*Gabar2*), the metabotropic glutamate receptor (*Grm5*), dopamine receptor 1, corticotropin-releasing factor receptor 1 (*Crhr1*), neuropeptide Y receptor 1 (*Y1*) and its ligand neuropeptide Y (*Npy*). Although, gamma-aminobutyric acid receptor 1b is not affected by either of the treatments and expression of the glucocorticoid receptor *Nr3c1* was only affected by HFD treatment, we can say that overall neurotransmission is changed by dietary intervention during adolescence. This is in line with results published in literature which has shown that changes in the microbiota results in altered neurotransmission. For example, the administration of a single bacterium, *Lactobacillus rhamnosus*, results in altered gene expression of

Gabra2 in the amygdala (Bravo et al., 2011) and is known to be changed drastically in the amygdala of germ free mice (Hoban et al., 2018). Overall, it can be concluded that the gene expression profile of the amygdala is drastically and long-lastingly changed following dietary intervention in adolescence.

In contrast to the amygdala which matures much faster, the prefrontal cortex still develops during adolescence. As it was shown previously that the microbiota affects myelination in the prefrontal cortex (Gacias et al., 2016; Hoban et al., 2016), we also analysed whether dietary intervention could alter gene expression of myelination-related genes. Indeed, HFD induced increased expression of Plp1, which could result in abnormal myelination in the prefrontal cortex (Karim et al., 2007) and therefore affect emotional learning. However, as myelination-related genes were only marginally changed by the dietary intervention and subsequent changes in gut microbiota composition we speculate that gene expression in the prefrontal cortex is not as drastically changed as in the amygdala. Although this data points towards a role of the gut microbiota in the regulation of gene expression in the amygdala and the prefrontal cortex, we cannot fully exclude the influence of the diet on its own on these parameters. Further studies will be needed to fully disentangle the role of the gut microbiota on long-lasting changes of gene expression during the adolescent period.

However, even though dietary intervention had long-lasting effects on the microbiota composition and gene expression, no long-lasting effects on physiological readouts or behaviour were observed. Different reasons might explain the lack of these effects. First, we cannot rule out that the lack of translatability of changes in the microbiota composition and gene expression to behavioural readouts are due to the selection of the test battery we used. We have focused on amygdala dependent tests, which have been shown to be affected by changes or the lack of microbiota (Cowan et al., 2018; Hoban et al., 2018; Stilling et

al., 2018). However, dietary intervention or the gut microbiota of high fat diet fed mice are often linked to changes in depressive-like and exploratory behaviour, as well as cognitive abilities (Bruce-Keller et al., 2015; Almeida-Suhett et al., 2017), which were not specifically tested in depth in this study. Furthermore, studies have shown, that behavioural effects at least in part can be reversed when switching back to normal chow following a high fat diet intervention (Sobesky et al., 2014; Boitard et al., 2016; Carlin et al., 2016) and 21 days is a relatively short time of diet exposure and brain changes in the adolescent brain can continue through to P60, which could account for the lack of behavioural differences in adulthood.

Overall, this study highlights the importance of the microbiota during the developmental period in shaping its host microbiota and brain. The results presented here support the need for further studies investigating the effect of dietary intervention during the adolescent period and their long-term effects on the microbiota, gut, immunity, brain and behaviour. Given that a dietary pattern high in fat and sucrose is associated with increased risk of mental health problems in adolescents (Oddy et al., 2018) it is important to determine to which degree the changes persists and to which extent they can be opposed by dietary intervention to prevent vulnerable phenotypes for mental health disorders. Furthermore, as it is difficult to fully disentangle the effects of the dietary intervention itself from the effects of altered gut microbiota composition during the adolescent period, it will be useful for future studies to study these effects utilising different factors to alter the microbiota such as antibiotics. Moreover, future studies should also investigate the underlying mechanisms underpinning such changes with a focus on differentiating between immune, humoral and neuronal communication routes (Cryan et al., 2019). Given that the vagus nerve is so important in such communication (Fulling et al., 2019) the inclusion of experiments on vagotomised animals will also be a useful approach.

Chapter 3 Targeting the Microbiome with Perturbations and Psychobiotics

3.1 Microbiota from Young Mice Selectively Counteracts the Effects of Aging Across the Microbiome-Gut-Immune-Brain Axis

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3.1.1 Introductory paragraph

The gut microbiota is increasingly recognized as an important regulator of host immunity and brain health. The aging process yields dramatic alterations in the microbiota, which is linked to poorer health and frailty in elderly populations. However, there is limited evidence for a mechanistic role of the gut microbiota in brain health and neuroimmunity during aging processes. Therefore, we conducted fecal microbiota transplantation (FMT) from either young (3-4 months) or old (19-20 months) donor mice into aged recipient mice (19-20 months). Transplant of a microbiota from young donors reversed aging-associated differences in peripheral and brain immunity, as well as the hippocampal metabolome of aging recipient mice. Finally, the young donor-derived microbiota attenuated selective age-associated impairments in cognitive and social behavior when transplanted into an aged host. Our results reveal that the microbiome may be a suitable therapeutic target to promote healthy aging.

3.1.2 Main Body

Aging triggers metabolic and immune alterations that lead to perturbation of brain function and behavior, including impairments in hippocampal-associated cognitive functioning and social behavior (Matt and Johnson, 2016; Bettio et al., 2017; Boehme et al., 2019; Boyer et al., 2019). Notably, the gut microbiota, encompassing the population of trillions of micro-organisms, undergoes a parallel community shift, which has been correlated to changes in host frailty and cognition (Claesson et al., 2012; Jackson et al., 2016; Jeffery et al., 2016; Cryan et al., 2019; Ghosh et al., 2020).

Animal models have shown specific roles for the microbiota in shaping hallmarks of aging in the gut (Stebegg et al., 2019; Donaldson et al., 2020). Moreover, the consequences of an elderly-associated microbiota on a young host showing alterations in host immunity, neurogenesis, and cognition (Fransen et al., 2017; Kundu et al., 2019; D'Amato et al., 2020; Li et al., 2020). Interestingly, transferring microbiota from young fish (African turquoise killifish) into middle-aged fish improves lifespan and motor behavior (Smith et al., 2017). However, it is completely unknown if microbiota from young donors can restore aging-associated impairments in mammals.

To determine if fecal microbiota transplantation (FMT) from young mice can ameliorate aging-induced neurocognitive and immune impairments, we collected fecal microbiota from naïve young mice (3-4 months) and transplanted this into aged mice (Aged yFMT, 19-20 months). A separate group of aged mice received fecal microbiota from naïve old mice to control for handling during FMT administration (Aged oFMT, 19-20 months). To allow aging-associated comparisons, naïve young mice received the same yFMT mixture (Young yFMT). We found aging-associated differences in microbiota (Fig. 3.1-1, Supplementary Table 1, 2), immunity (Fig. 3.1-2, Extended Data Fig. 2, 3), hippocampal neurogenesis (Extended Data Fig. 2), hippocampal metabolomics (Fig. 3.1-3, Supplementary Table 3), and behavior (Fig. 3.1-4, Extended Data Fig. 5) – some, but not all, of which were attenuated by

microbiota transplantation from a young mouse into an aged host. Our research offers the intriguing possibility that a microbiota from a young individual may have beneficial effects when given to an aged host.

3.1.2.1 Age-associated Changes in the Gut Microbiota are Shifted Following FMT

To investigate the impact of aging on fecal microbiota, we analyzed baseline fecal microbiomes of young and aged mice using 16S rRNA amplicon sequencing. We reassessed composition and diversity after 4 weeks of FMT to examine engraftment and colonization dynamics of the FMT (Fig. 3.1-1a). Pre-FMT, we found clear differences between young and aged mice in beta-diversity. However, following FMT, differences in beta-diversity were no longer significant (Fig. 3.1-1b). No temporal or intergroup alpha-diversity changes were significant using Simpson or Shannon indices. The fact that only richness, measured by Chao1, dropped significantly following FMT in all groups (Fig. 3.1-1c), indicates that evenness was retained among the reduced number of taxa post-FMT. Twenty genera were significantly changed following FMT (Fig. 3.1-1d). Some of these temporal differences appeared congruent between aged mice regardless of treatment, indicating potential age-dependent responses regardless of the content of the FMT. On the other hand, certain genera, including *Enterococcus*, were altered in aged mice, but transitioned towards young mouse abundance following yFMT exclusively (Fig. 3.1-1e).

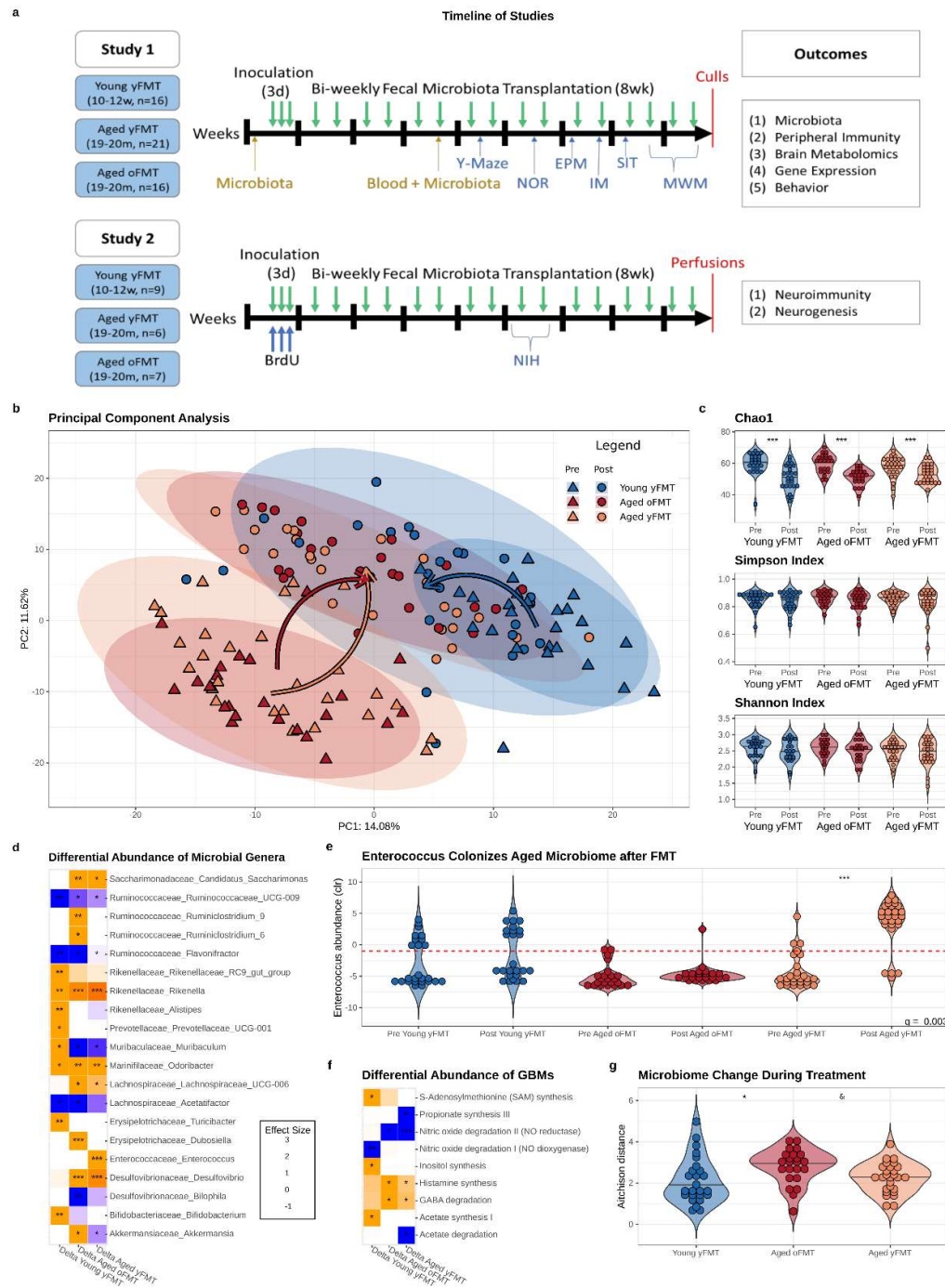


Fig. 3.1-1 – FMT Alters the Microbiota on a Taxonomic and Functional Level (a) Overview of the experimental design and the study timeline. Vertical bars represent one week. Behaviors conducted include Y-Maze, Novel Object Recognition Test (NOR), Elevated Plus Maze (EPM), Intestinal Motility (IM), Three-Chamber Social Interaction Test (SIT), Morris Water Maze (MWM), Observation Battery (OB), and, in Study 2, the Novelty-Induced Hypophagia Test (NIH). BrdU indicates intraperitoneal injection of bromodeoxyuridine. **(b)** Principal Component Analysis showing the effects of FMT on the fecal microbiome in young and aged mice in terms of beta-diversity as measured

in Aitchison distance. Arrows indicate the trajectory per group following its respective treatment. Ellipses show the 95% confidence intervals per group. WD*: $p < 0.001$. (c) Violin plots displaying the effects of FMT on young and aged mice in terms of alpha-diversity. Black horizontal lines in violin plots depict the medians. The Chao1 index showed a decrease after FMT; two-way ANOVA: ($F(1,144)= 50.1$ $p<0.001$). *** $p<0.001$. No effects were found in the Shannon or Simpson index. (d) Heatmap showing genera differentially altered by FMT. Color depicts effect size, with blue (negative) indicating higher abundances pre-treatment and red (positive) indicating higher abundances post-treatment. * $q < 0.1$, ** $q < 0.01$, *** $q < 0.001$. Comprehensive statistical results can be found in Supplementary Table 1. (e) Violin plots showing *Enterococcus* abundance is restored to young levels in old mice after yFMT but not after oFMT. Black horizontal lines in violin plots depict the medians. Y-axis shows CLR-transformed abundance. Dashed horizontal red line depicts a threshold for estimated abundance of 0 (Mann-Whitney U test: $p<0.001$, $q<0.001$, $e=1.48$). (f) Heatmap showing gut-brain modules (GBMs) differentially altered by FMT. Color depicts effect size, with blue (negative) indicating higher abundances pre-treatment and red (positive) indicating higher abundances post-treatment. * $q < 0.1$, ** $q<0.01$, *** $q<0.001$. Comprehensive statistical results can be found in Supplementary Table 2. (g) Violin plots showing the differences in Aitchison distance traveled per microbiome after treatment. Black horizontal lines in violin plots depict the medians. Aged oFMT group changed more than Young yFMT ($F(72)=3.82$ $p=0.026$). Tukey's HSD: Young yFMT vs Aged oFMT: $p=0.03$. Young yFMT vs Aged yFMT: $p=0.88$. Aged yFMT vs Aged oFMT: $p=0.082$. * $p<0.05$, & $p<0.1$.

Functionally, we found significant changes in nine gut-brain modules (GBMs)(Valles-Colomer et al., 2019) following FMT (Fig. 3.1-1f). Interestingly, propionate synthesis III and acetate degradation were reduced in Aged yFMT, but not in Aged oFMT mice, suggesting differences in the metabolism of short-chain fatty acids (SCFA), which are involved in gut-brain communication and aging(Unger et al., 2016; Dalile et al., 2019). We found that Aged oFMT microbiota composition had changed significantly more than Young yFMT in terms of Aitchison distance post FMT, while no significant change was observed between Aged yFMT and Young yFMT (Fig. 3.1-1g).

3.1.2.2 yFMT Modulates Age-Associated Mesenteric Lymph Node and Hippocampal Immune Changes

The gut microbiota is a key regulator of host immunity especially in aging (Fransen et al., 2017; Thevaranjan et al., 2017). Moreover, the immune system influences hippocampal-associated cognitive and social behavior (Filiano et al., 2016; Mohle et

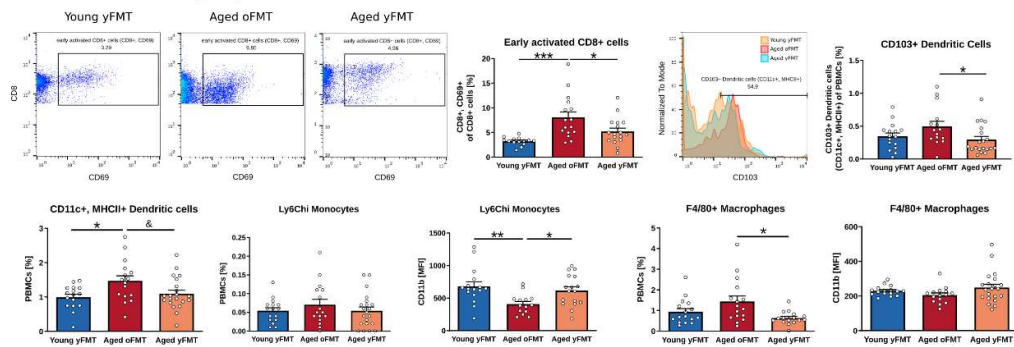
al., 2016; Cruz-Pereira et al., 2020). Thus, the immune system may be an important link between alterations in the gut microbiota and potential effects on the brain in aging.

To determine if aging-invoked immune alterations can be ameliorated by FMT from a young donor, we characterized innate and adaptive immunity in the mesenteric lymph nodes (MLNs) and circulation. Aging triggered a substantial increase in early activated CD8⁺ T-Cells in MLNs, which was reversed by yFMT (Fig. 3.1-2a). In contrast, memory CD8⁺ T-cells (CD44^{hi}) were unaffected (Extended Data Fig. 3a), perhaps due to the low turnover rate of memory CD8⁺ T-cells (Baliu-Pique et al., 2018). Furthermore, CD103⁺ dendritic cells, which are tightly linked to CD8⁺ T-cell activation (Joffre et al., 2012) and sensitive to changes in the microbiome (Tan et al., 2016), were reduced in Aged yFMT mice compared to Aged oFMT mice (Fig. 3.1-2a). yFMT into aged mice decreased the proportion of F4/80⁺ macrophages in MLNs and rescued the decrease of the activation marker CD11b on Ly6C^{hi} monocytes (Fig. 2a). Despite strong effects of aging on innate and adaptive immunity in the circulation (Extended Data Fig. 3c, d), yFMT only trended towards decreasing the aging-associated rise of marker CD11b on Ly6G⁺ neutrophils (Fig. 3.1-2b) (Uhl et al., 2016) while rescuing the aging-associated rise in peripheral IL-10 (Fig. 3.1-2b). However, yFMT did not reverse aging-associated reductions in IFN γ and IL-5, which are both predominantly produced by T-cell populations, implying a stage of immunosenescence (Fig. 3.1-2b). This indicates that yFMT into an aged host may selectively modulate peripheral immunity. The mesenteric lymph node immune profile was particularly sensitive to FMT-induced changes in the aged host.

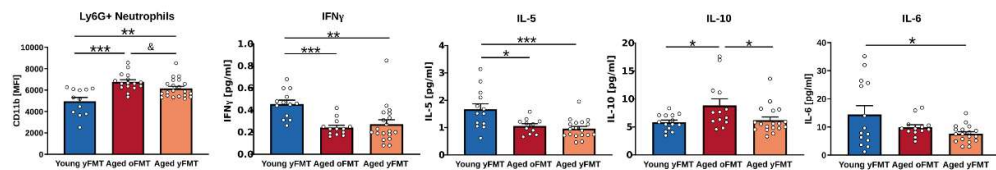
Given the relationship between the gut microbiota, neuroinflammatory processes, and brain plasticity (Erny et al., 2015; Mohle et al., 2016; Thion et al., 2018; van de Wouw et al., 2019), we characterized microglia, the brain's resident macrophages, in the hippocampus, a brain region critical for cognition. Microglia are essential for regulating cellular aspects of cognition, supporting neuroplasticity (Rogers et al.,

2011; Elmore et al., 2018), and responding to various signals, including cytokines. Higher populations of activated microglia, distinguished by enlarged somas, are prominent in neurodegenerative conditions (Tay et al., 2017; Hickman et al., 2018). Aged oFMT mice showed substantial enlargement in microglia cell soma size, which was further pronounced when microglia somas were classified as 'large' or 'small' (Fig. 3.1-2c) (Kozareva et al., 2019). This phenotype was reversed by yFMT (Fig 3.1-2c). However, we did not observe changes in microglia complexity, or maximum or average branch length (Extended Data Fig. 2c).

a Mesenteric Lymph Nodes



b Circulation



c Hippocampal Neuroimmunity Microglia

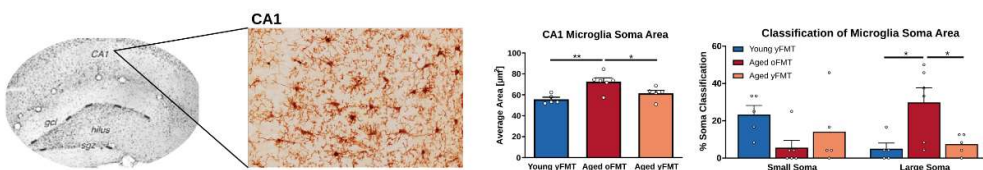


Fig. 3.1-2 - FMT from Young to Aged Mice Modulate Peripheral Immunity and Hippocampal Neuroimmunity (a) Immune profile in mesenteric lymph nodes (MLN) was assessed by Flow Cytometry at the end of the study. Differences were detected in both early activated CD8⁺ T-cells (CD69⁺) ($F(2,44)=9.418$, $p<0.001$) and CD103⁺ dendritic cells (DC) ($H(3)=7.836$, $p=0.02$) in MLNs. Interestingly, yFMT into aged mice reversed ($p=0.022$) the age-associated increase ($p<0.001$) in early activated CD8⁺ T-cells, while Aged yFMT showed reduced levels of CD103⁺ DCs compared to Aged oFMT ($p=0.023$). (b) Immune profile in circulation was assessed before mice were assessed for behavior. Distinct marker expression change, assessed by median fluorescence

intensity (MFI), for the activation marker CD11b on Ly6G⁺ neutrophils ($F(2,46)=12.53$, $p<0.001$), were found with an increased expression in Aged oFMT (CD11b: $p<0.001$) which tended to be rescued in Aged yFMT (CD11b: $p=0.0645$). Cytokine levels were assessed in the circulation. Notably, both IFN γ and IL-5 decreased in both age groups ($F(2,42)=9.331$, $p<0.001$, and $F(2,39)=8.758$, $p<0.001$) respectively) while an age-associated increase in IL-10 in Aged oFMT ($F(2,42)=4.357$, $p<0.001$, post-hoc: $p=0.03$) was found which was counteracted in Aged yFMT ($p=0.033$). Interestingly, Aged yFMT exhibited decreased IL-6 compared to Young yFMT ($F(2,42)=3.768$, $p=0.031$, post-hoc: $p=0.027$). (c) Hippocampal neuroimmunity was assessed by examining microglia soma area ($F(2, 13)=8.418$, $p=0.005$). There was a significant increase in microglia soma size in Aged oFMT mice compared to Young yFMT mice ($p=0.005$), which was reversed in Aged yFMT mice compared to Aged oFMT ($p=0.042$). When microglia were further classified as having a 'large' or 'small' soma ($F(2, 13)=6.328$, $p=0.012$), Aged oFMT mice had significantly more microglia classified as 'large' compared to Young yFMT ($p=0.022$) and Aged yFMT ($p=0.264$). Mean \pm SEM. $n = 12-21$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ & $p<0.1$.

In addition to supporting brain immunity, microglia regulate hippocampal neurogenesis (Gemma et al., 2010; Sierra et al., 2010), a key activity in some learning and memory processes (Sahay et al., 2011; Anacker and Hen, 2017), which is hindered in aging (Klempin and Kempermann, 2007). Intestinal microbiota can alter hippocampal neurogenesis, potentially contributing to age-associated disruptions in neurocognition (Ogbonnaya et al., 2015; Kundu et al., 2019). Using another cohort of animals (Fig. 3.1-1a) we confirmed a decrease in the number of surviving newly-born hippocampal neurons in aged mice (Extended Data Fig. 2a). But this was not prevented with yFMT.

3.1.2.3 The Aged Hippocampal Metabolome is Sensitive to the Rejuvenating Effects of yFMT

Given the aging-associated changes in the brain and the influence of gut microbiota on hippocampal function (Mohle et al., 2016), we examined whether FMT from young to aged mice shaped the hippocampal metabolome.

Aging induced significant differences in hippocampal metabolites, with aged and young mice clustering separately (Fig. 3.1-3a, 3.1-3c). Notably, 35 metabolites altered

in aging were restored towards pre-aged levels by yFMT (Fig. 3.1-3b, 3.1-3d and Supplementary Table S3), resulting in Aged yFMT mouse hippocampal metabolomes clustering between aged and young individuals (Fig. 3.1-3c). Notably, GABA and N-glycolylneuramate were among those restored by yFMT. These metabolites play critical roles in cognition and brain plasticity (Wang, 2012; Fritschy and Panzanelli, 2014). In line with previous research (Hunsberger et al., 2020), arginine increased with aging, and is linked to the nitric oxide pathway and neurodegeneration (Wu and Morris, 1998; Malinski, 2007); its reversal by yFMT into aged mice proposes possible neuroprotective effects of yFMT on the aged brain. Functionally, these restored metabolites are enriched in six metabolic pathways (Fig. 3.1-3e), predominantly related to amino acid metabolism, which is critical for healthy cognition (Canfield and Bradshaw, 2019), and aminoacyl tRNA biosynthesis, which is crucial for proper functioning of the brain (Schaffer et al., 2019).

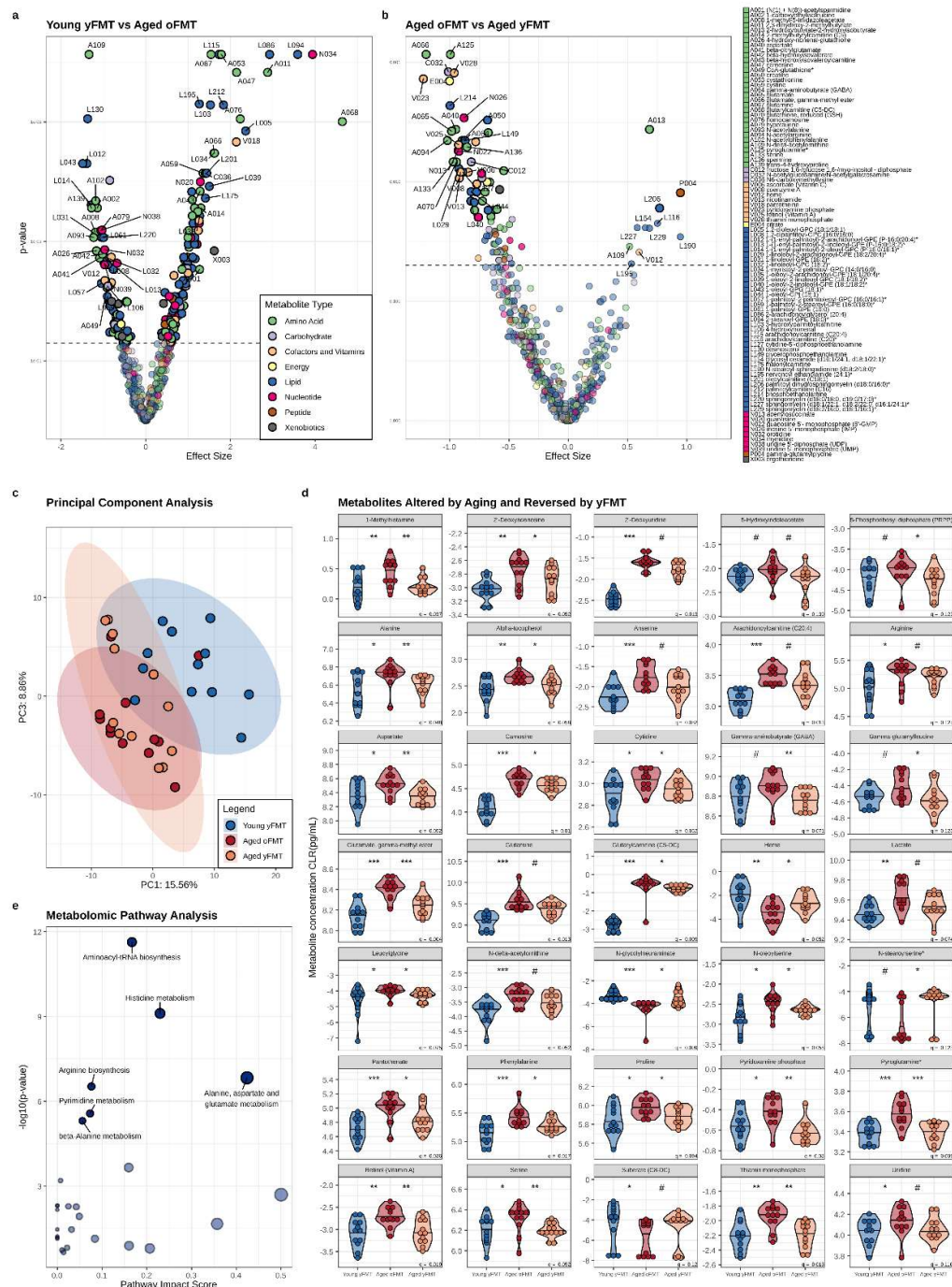


Fig. 3.1-3 - FMT from Young to Aged Mice Shapes the Hippocampal Metabolome
 Volcano plots depicting the effect of aging (a) and the difference between oFMT and yFMT (b) in the hippocampal metabolome. Metabolites on the 5% extremes of effect sizes of differential abundance are labelled. Horizontal dashed lines represent $p=0.05$. Comparisons with $p>0.05$ or $q>0.2$ are depicted as smaller and more transparent. Comprehensive statistical results including the full legend for the abbreviations can be found in Supplementary Table 2. (c) Principal Component Analysis showing the effects of FMT on the hippocampal metabolome in young and aged mice in terms of

Aitchison distance. Ellipses show the 95% confidence intervals per group. PERMANOVA: $p < 0.001$. (d) Violin plots representing the 35 metabolites that were significantly altered by aging and restored towards young levels after yFMT. Black horizontal lines in violin plots depict the medians. Y-axis shows CLR-transformed metabolite concentrations. Q-values can be seen in the bottom-right corners of every figure. Significance symbols represent Mann-Whitney U-test post-hoc tests: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.1$. Comprehensive statistical results can be found in Supplementary Table 2. (e) Scatterplot showing the results of the MetaboAnalyst Pathway analysis. Labeled, dark-blue points depict the pathways that withstood FDR at a 0.1 threshold.

3.1.2.4 yFMT Counteracts Hippocampal-Dependent Cognitive and Social Behavioral Changes in Aging.

Alterations in the gut microbiome have been widely linked to changes in behavior, including hippocampal-dependent cognition (Cryan et al., 2019), which declines during the aging process (Smith et al., 2000; Scheff et al., 2006; Koh et al., 2014). Thus, we examined if yFMT rescued aging-induced, hippocampus-associated behavioral abnormalities in aged mice in a series of cognitive tasks. Consistent with the extant literature, aging was associated with an increase in the latency to enter the target quadrant in the Morris Water Maze probe trial. However, this was attenuated in aged mice that received FMT from a young donor (Fig. 3.1-4c), indicating that yFMT may improve aging-associated impairments in long-term spatial memory. Notably, there were no differences in locomotor activity between groups (Fig 3.1-4e).

We also assessed short-term working memory and short-term recognition memory using the Y-Maze and Novel Object Recognition (NOR) tests. While there were no significant aging-induced impairments in either spontaneous alternation behavior in the Y-Maze or the novel object recognition index (Fig. 3.1-4a, b), Aged oFMT mice tended to interact less with objects in the NOR test (Fig. 3.1-4b). Furthermore, in the Three-Chamber Social Interaction Test, Aged oFMT mice spent significantly less time interacting with a social partner than their Young yFMT counterparts (Fig. 3.1-4d). Both aging-associated deficits in environmental interaction were rescued by FMT from young donors (Fig. 3.1-4b, d). Overall, these data suggest that yFMT can rescue specific aspects of aging-induced impairments in behavior.

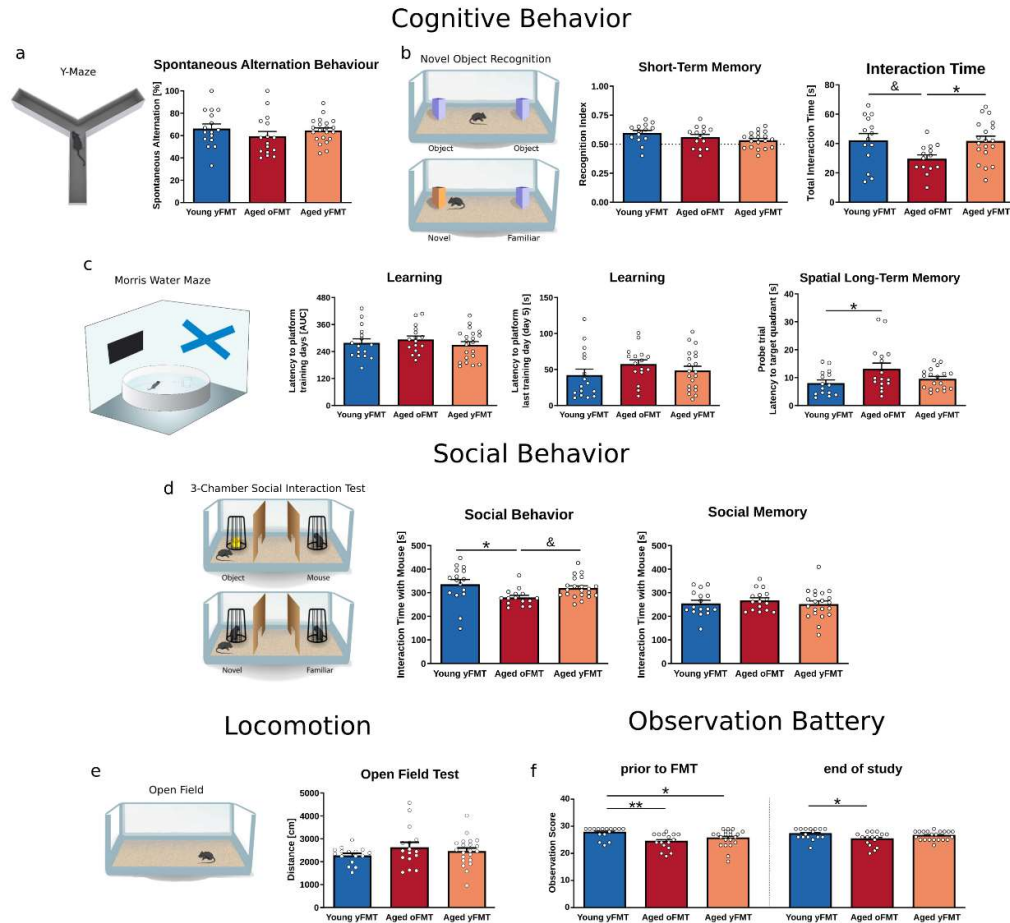


Fig. 3.1-4 - FMT from Young to Aged Mice Influence Behavior (a) Short-term memory assessed by spontaneous alternation behavior (Y-maze). No significant differences were observed between groups. (b) Short-term memory and exploration in the Novel Object Recognition (NOR) test. While long-term memory in the NOR was not significantly different across groups, there were significant differences in exploration of the objects ($F(2,44)=3.388$, $p=0.028$), with aged mice receiving yFMT (Aged yFMT) showing an increased exploration activity compared to Aged oFMT ($p=0.043$). (c) Learning and memory in Morris Water Maze: Latency-to-find platform over five training days summarized as area under-the-curve (AUC) displays the learning efficacy to find the platform. Interestingly, neither age nor FMT did affect learning efficacy. Subsequently, we assessed long-term memory in the probe trial 24 h after the last training day by measuring the latency to find the target quadrant. The treatment significantly altered the time to reach the target quadrant ($F(2,47)=3.357$, $p=0.043$) with Aged oFMT ($p=0.041$) needed a longer time to allocate the target quadrant which was not seen in aged mice which received yFMT. (d) Social behavior and memory: Treatment affected social behavior with respect to the time the test mouse interacted with a con-specific mouse (over an object) in the second phase of the 3-chamber social interaction test ($F(2,49)=4.168$, $p=0.021$) while interaction with a novel mouse (over the now familiar mouse) in the third phase of the 3-Chamber

*Social Interaction Test remained unaffected by the treatment. Post-hoc analysis indicated decreased interaction with a con-specific mouse in Aged oFMT compared to Young yFMT ($p=0.022$) which trended to be rescued by yFMT into aged mice ($p=0.077$). (e) Locomotion: Locomotion was unaltered by age (Aged oFMT) or FMT from young to aged mice (Aged yFMT). (f) Observation Battery: Bar plots show observation battery score prior to FMT compared to end of study. Both aged groups showed a decreased score prior to FMT compared to Young yFMT ($H(3)=13.33$, $p=0.001$; post-hoc: Aged oFMT $p=0.001$, and Aged yFMT $p=0.038$, respectively). In contrast, Aged yFMT did not more show a significant difference ($p=0.46$) at the end of the study while Aged oFMT retained a decreased score compared to Young yFMT ($H(3)=6.146$, $p=0.046$; post-hoc: $p=0.04$). Mean \pm SEM. $n = 14-21$, $*p<0.05$, $**p<0.01$, & $p<0.1$.*

3.1.3 Conclusion

Here, we demonstrate, for what is to our knowledge the first time, that transplanting microbiota from healthy, young male mice into aged male mice can significantly attenuate aging-associated deficits in cognitive behavior. Upon investigating potential mechanisms for how the intestinal microbiota may orchestrate these improvements, we found specific aspects of peripheral immunity and neuroimmunity were restored following yFMT into aged male mice. Moreover, we uncovered amelioratory effects of yFMT on the aging hippocampal metabolome, which coincided with the improvements in behavior. While specific aging-associated deficits in behavior, immunity, or neurogenesis were not restored by yFMT, this research provides fundamental evidence that the gut microbiota should be considered as a therapeutic target for treating aspects of aging-associated decline in hippocampus-related function.

3.1.4 Methods

3.1.4.1 Animals

Male young adult C57BL/6 mice ($n=25$; Envigo, Cambridgeshire, UK; 10-12 weeks) and aged C57BL/6 mice ($n=50$; Charles River, UK, 19-20 months) were used in this study. All experiments were performed in accordance with European guidelines following approval by University College Cork Animal Ethics Experimentation

Committee (AE19130/P052). Animals were habituated to the animal facility for at least four weeks before experiments started and were kept under a 12-hour light/dark cycle, with a temperature of 21 ± 1 °C and humidity of $55 \pm 10\%$. Food and water were given *ad libitum*.

3.1.4.2 Fecal collection and fecal microbiota transplant (FMT)

For all fecal material collection, mice were placed in an empty plastic cage, free of bedding and sterilized with 70% ethanol, until defecation. For future microbiome analysis, fecal pellets were immediately collected and snap-frozen on dry ice, then stored at -80 °C until sequencing.

To collect material for FMT, a separate cohort of naive young adult C57BL/6 mice (n = 40, Envigo, Cambridgeshire; 12-15 weeks at collection, group-housed), and the same aged mice defined above, supplemented with additional naive old mice to ensure enough collection volume (n = 83, 18.8-20 months at collection, group-housed), were used, prior to the start of the experimental timeline. Fecal pellets were collected fresh and immediately transferred to an anaerobic hood, where they were pooled per group to ensure enough volume. Fecal material was homogenized in reduced sterile phosphate buffered saline (PBS) with 20% glycerol (w/v), and filtered through a 70 µm strainer to remove large particles before being aliquoted and frozen at -80 °C. To ensure enough volume of FMT material without risking potential cross contamination, collection was spaced out over six to nine collection days per group, one group at a time. Prior to FMT, equal aliquots from every collection day were pooled together to consistently generate enough volume.

Fecal microbiota transplantation (FMT) was performed by oral gavage bi-weekly (100 µl of 100 mg/ml homogenized fecal slurry). Briefly, mice were gently scruffed, and the fecal slurry was administered through an oral gavage needle (23G). FMT occurred once per day for the first three days to encourage microbiota engraftment, then twice per week thenceforth.

3.1.4.3 Study design and experimental timeline

Aged mice were randomly delegated into one of two groups, balanced by weight: aged mice receiving FMT from young donor mice (Aged yFMT), and to control for handling, aged mice receiving FMT from old donor mice (Aged oFMT). Young mice were given FMT from young mice (Young yFMT) to allow for inter-age comparisons. To ensure appropriate engraftment of microbiota, and to allow for potential microbiota-driven neurocognitive and behavioral effects, mice were orally gavaged with FMT once per day for the first three days, then twice weekly thenceforth in line with previous research (Bruce-Keller et al., 2015).

Baseline fecal samples were collected prior to the start of the study and after four weeks of FMT inoculation to assess the effects of FMT on recipient fecal microbiota prior to the start of the behavioral battery. Following four weeks of FMT, blood was collected, and flow cytometry was run to assess markers of mesenteric and peripheral immunity accompanied with cytokines measurements (see flow cytometry methodology under 3.6, and cytokine assessment under 3.10). Mice then underwent a series of behavioral tests designed to assess a variety of locomotor, social, cognitive and anxiety-like parameters, including the following: Y-Maze, Novel Object Recognition, Elevated Plus Maze, 3-Chamber Social Interaction Test (Sociability and Social Memory), and the Morris Water Maze. Finally, mice were euthanized via decapitation, and blood and tissues immediately harvested for further analysis. See experimental timeline Fig. 1a.

Aged and young mice were monitored throughout the study through a dedicated observation battery which comprises different aspects of health including general appearance, physical characteristics and sensorimotor reflexes (Roux et al., 2005; O'Leary et al., 2016) (see Extended Data Fig. 5).

An additional study was performed following a similar design as above to allow for visualization and further investigation potential mechanisms of action, including microglia and adult hippocampal neurogenesis. Herein, mice were treated once per day with 150 mg/kg Bromodeoxyuridine (BrdU; Sigma-Aldrich, cat. no. 59-14-3) diluted to 20 mg/ml in sterile saline via intraperitoneal injection during the first three FMT inoculation days to label proliferating cells. Following four weeks of FMT inoculation, the Novelty-Induced Hypophagia Test was performed. Finally, mice were anesthetized with 90 mg/kg pentobarbital and tissue fixed via transcardial perfusion with chilled sterile saline for two minutes, followed by eight minutes of 4% paraformaldehyde (PFA). Brains were harvested and transferred into 4% PFA overnight, then transferred to 15% sucrose overnight, and finally 30% sucrose for 24 hours before being snap-frozen in isopentane and stored at -80 °C.

3.1.4.4 DNA extraction for 16S rRNA amplicon microbiota analysis

DNA was extracted using the QiaAMP Power Fecal Pro kit (Qiagen) according to the manufacturer's instructions. DNA concentration was normalized and 16S rRNA amplicon libraries were prepared using primers to amplify the V3-V4 region of the bacterial 16S rRNA gene, with Illumina adapters incorporated as described in the Illumina 16S rRNA Metagenomic Library Preparation guide, with the exception that 30 amplification cycles were used. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. The pooled libraries were assessed using an Agilent high sensitivity DNA kit and examined by quantitative PCR (qPCR) using the Kapa Quantification kit for Illumina (Kapa Biosystems, USA) according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced (2 × 300 bp) on the Illumina MiSeq platform.

3.1.4.5 16S Microbiota analysis and bioinformatics

Paired-end reads were pre-filtered based on a quality score threshold of >28 and trimmed, filtered for quality and chimaeras using the DADA2 library in R (version

3.6.3)(Callahan et al., 2016). QC was done using the fastqc program in Ubuntu 18.04. Samples with fewer than 10,000 reads after filtering were discarded. Taxonomy was assigned with DADA2 against the SILVA SSURef database release v138(Quast et al., 2013). Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. Amplicon Sequence Variants (ASVs) were aggregated at genus level. Those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as non-zero in 10% or fewer of total samples. After filtering, 86 different genera were left in total over all microbiome samples.

Microbiota bioinformatics were done in R (version 3.6.3) with the Rstudio GUI (version 1.2.5033). Principal component analysis was performed on center log-ratio transformed (clr) values using the ALDEx2 library(Fernandes et al., 2014). The number of permutations was always set to 1000. The iNEXT library was used to calculate alpha diversity using the Chao1, Shannon and Simpson indices. Beta-diversity was assessed in terms of Aitchison distance and visualized via principle component analysis(Hsieh et al., 2016). Wd*(Hamidi et al., 2019) followed by a pairwise post-hoc and Bonferroni was used to find structural differences between treatments on a compositional level. The degree of change per mouse through time was calculated in terms of Aitchison distance, using one-way ANOVA followed by Tukey's test. Degree of change in the microbiome after treatment, or volatility, was calculated as Aitchison distance. The Piphillin web server was used to infer the functional metagenome in terms of KEGG-orthologues(Iwai et al., 2016). Using these KEGG-orthologues, Gut-Brain Modules and Gut-Metabolic Modules were calculated using the omixerRpm R library(Valles-Colomer et al., 2019). Differential abundance of microbial genera as well as functional modules was calculated using the Wilcoxon signed-rank test implementation in the ALDEx2 library. To correct for multiple testing (FDR) in tests involving microbiota features, the internal ALDEx2 implementation of the Benjamini-Hochberg (BH) post-hoc procedure was performed with a q-value of 0.1 as a cut-off. All custom R scripts are available online at

<https://github.com/thomazbastiaanssen/Tjazi> (Bastiaanssen, 2018). Microbiota figures were generated using ggplot2.

3.1.4.6 Flow cytometry

To assess immunity following four weeks of intervention with FMT, before mice were subjected to behavioral assessments, blood sample preparation was done as previously described with minor modifications (van de Wouw et al., 2020). Briefly, blood was collected by tail tipping using Eppendorf tubes containing 2.5 μ L 3% EDTA solution to prevent blood clotting. Blood was resuspended in each 10 mL home-made red blood cell lysis buffer (15.5 mM NH₄Cl, 1.2 mM NaHCO₃, 0.01 mM tetrasodium EDTA diluted in deionized water) for three min. Blood samples were subsequently centrifuged (1500 \times g, 5 min), plasma taken for cytokine analysis using the Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, Maryland, USA) (see 3.10 for full details of methodology), and cells resuspended in PBS containing 1:1000 FVS780 (PE-Cy7) (BD Biosciences, cat. no. 565388) and incubated for 15 min at RT to distinguish live from dead cells. Subsequently, samples were centrifuged (1500 \times g, 5 min) and each aliquot (two per sample) resuspended in 50 μ L BV staining buffer (BD Biosciences, cat. no. 563794). All subsequent procedures were conducted on ice / at 4°C. For the staining procedure, 5 μ L of FcR blocking reagent (Miltenyi, cat. no. 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies for extracellular staining to investigate (a) innate immune system properties focused on monocytes / neutrophils, and (b) adaptive immune system properties focused on T-cells (see Table 1) and incubated for 30 min on ice. Samples for panel a (monocytes / neutrophils populations) were subsequently washed in staining buffer and fixed in 4% PFA for 30 min on ice, whilst samples for panel b (T-cells populations) underwent intracellular staining using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, cat. no. 00-5523-00), according to the manufacturer's instructions, together with FoxP3-APC (Thermo Fisher Scientific, cat. no. 17-5773-82). Fixed samples were subsequently resuspended in the staining buffer and analyzed the subsequent day using the BD FACSCelesta. Data was analyzed using FlowJo (version 10). Target populations were

normalized to the number of live cells or the respective parent population. Gating strategy is depicted in Extended Data Fig. 4.

To assess gut-associated immunity at the end of the study, mesenteric lymph nodes (MLNs) were carefully dissected and processed for Flow Cytometry as previously described (van de Wouw et al., 2020). Briefly, MLNs were transferred onto a 70 μ m strainer and disassembled using the plunger of a 1-mL syringe. The strainer was subsequently washed with 10 mL media (RPMI-1640 medium with L-glutamine and sodium bicarbonate, supplemented with 10% FBS (Sigma, cat. no. F7524I) and 1% Pen/strep (Sigma, cat. no. P4333), centrifuged and 3×10^6 cells were resuspended in 90 μ L staining buffer and split into three aliquots for the staining procedure. Samples were always kept on ice. For the staining procedure, 5 μ L of FcR blocking reagent (Miltenyi, cat. no. 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (see Table 2) for 30 min on ice followed by a washing step and final fixation using 4% PFA for 30 min on ice. Samples were analyzed the subsequent day using the BD FACSCalibur. Data was analyzed using FlowJo (version 10). The investigated cell populations were normalized to the number of obtained peripheral blood mononuclear cells (PBMCs).

Table 1

Marker	Conjugation	Volume per sample [μ L]	Company, number	Catalog
Innate immune system - Focus Monocytes / Neutrophils				
CD11b	VFITC	5	Miltenyi	130-109-290
CD11c	PE	2	Miltenyi	130-110-701

CD62L	PE-Cy7	2	Biolegend 104418
LY6G	PerCP-Vio700	5	Miltenyi 130-107-917
CD192 (CCR2)	APC	5	Miltenyi 130-108-723
MHC-II	BV421	0.5	Biolegend 107632
LY6C	BV605	2	Biolegend 128036
CX3CR1	BV786	2	Biolegend 149029
Adaptive immune system - Focus T-cells			
CD49b	FITC	5	Miltenyi 130-102-258
CD69	PE	5	Miltenyi 130-103-946
CD62L	PE-Cy7	2	Biolegend 104418
CD8a	PerCP-Vio700	5	Miltenyi 130-102-239
FoxP3	APC	5	Thermo Fisher Scientific

(intracellular staining)			17-5773-82
CD25	BV421	2	Biolegend 102034
CD4	BV605	0.5	Biolegend 100548
CD44	BV786	2	BD Biosciences 563736

Table 2

Marker	Conjugation	Volume per sample [μl]	Company, Catalog number
T-cell panel			
CD4	FITC	0.5	Thermo Fisher Scientific 11-0042-85
CD69	PE	5	Miltenyi 130-103-946
CD8a	PerCP-Vio700	5	Miltenyi 130-102-239
CD44	APC	5	Miltenyi 130-110-084
Monocyte/Macrophage/Neutrophil panel			

CD11b	FITC	2	Miltenyi 130-113-243
LY6C	PE	5	Miltenyi 130-102-391
LY6G	PerCP-Vio700	5	Miltenyi 130-107-917
F4/80	APC	5	Miltenyi 130-102-379
Dendritic cell panel			
CD103	FITC	5	Miltenyi 130-102-479
CD11c	PE	2	Miltenyi 130-110-701
CX3CR1	PerCP-Cy5.5	0.3	Biolegend 149010
MHC-II	APC	5	Miltenyi 130-102-139

3.1.4.7 Hippocampal Metabolomics

Mice were decapitated at the end of the study, the hippocampus carefully dissected, immediately snap frozen in liquid nitrogen and stored at -80 °C until use. For

metabolomics, the left hemisphere of the hippocampus was used. Hippocampal metabolomics were conducted at Metabolon using UHPLC/MS/MS.

Samples were prepared using the automated MicroLab STAR[®] system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap[®] (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Several controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples

were randomized across the platform run with QC samples spaced evenly among the injections.

For analysis, a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution was utilized. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μ m) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Raw data was extracted, peak-identified and QC processed using Metabolon's hard- and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities.

Peaks were quantified using area-under-the-curve. Biostatistics were done in R (version 3.6.3) with the Rstudio GUI (version 1.2.5033). Missing values were imputed by taking 95% of the minimum observed abundance per metabolite. Metabolites that were detected in less than 10% of samples were dropped from analysis. Principal component analysis was performed on centered log-ratio transformed (clr) values using the ALDEx2 library (Fernandes et al., 2014). Number of permutations was always set to 1000. PERMANOVA followed by a pairwise PERMANOVA was used to find structural differences between treatments on a compositional level. In order to find metabolites that were altered by aging and restored by yFMT, we first did a Mann-Whitney U-test between all yFMT samples and the Aged oFMT group, identifying metabolites where the Aged oFMT group was different from the two yFMT groups. Then we did pairwise Mann-Whitney U-tests in between both individual yFMT groups and the Aged oFMT group, to verify that both yFMT groups were indeed different from the Aged oFMT group levels individually. To correct for multiple testing in tests involving metabolomics features, Storey's q-value post-hoc procedure was performed with a q-value of 0.2 as a cut-off. Metabolites that were found to be altered in aging and restored by yFMT were mapped to their corresponding Human Metabolome Database (HMDB) identifier and subjected to pathway analysis using the MetaboAnalyst online pipeline, choosing the murine KEGG library as a reference. Custom R scripts are available online at <https://github.com/thomazbastiaanssen/Tjazi>. Metabolomics figures were generated using ggplot2.

3.1.4.8 Behavior

All behavioral tests were performed and scored by experimenters blinded to the treatment groups.

3.1.4.8.1 Spontaneous alternation in the Y-Maze

To access short-term working memory, spontaneous alternation behavior in the Y-maze tests hippocampal-dependent spatial memory and exploration and was carried out and analyzed as previously described (Scott et al., 2017). Briefly, mice were habituated to the room 30 min prior to testing, and behavior recorded for five minutes using a ceiling-mounted camera.

3.1.4.8.2 Novel Object Recognition

To assess short-term recognition memory, mice were subjected to the Novel Object Recognition (NOR) task as previously described with some modifications (Golubeva et al., 2017). Animals were habituated to the room 60 min before the test. On the pre-trial day, mice were habituated to the empty, open arena (40×45×45 cm, L×W×H) in two habituation phases (10 min each, with 3h time in between). On the following day, test mouse was exposed for 10 min to two identical objects placed in the corners of the arena (acquisition phase). 3 h later, one of the familiar objects was substituted with a novel object, and the animal could explore the objects for 10 min (retention phase). The test was conducted under dimmed lighting (15 lux). Animal behavior was video recorded; time spent in exploration of the objects was blindly scored in Ethovision version 15 (Noldus). Exploration behavior was defined as orienting the nose towards the object at a distance < 2 cm, or direct contact with the object. Discrimination index was calculated according to the formula: $(t[\text{novel}] - t[\text{familiar}]) / (t[\text{novel}] + t[\text{familiar}])$. Mice who interacted in sum less than 12 seconds with either the novel or the familiar object, were excluded from the analysis.

3.1.4.8.3 Open Field

The Open Field is a widely used assay to assess approach avoidance behavior, locomotor activity and the behavioral response to a novel context and was conducted as previously described with some modifications (van de Wouw et al., 2020). Briefly, mice were placed in an open arena (40×45×45 cm, L×W×H) and could explore the arena for 10 min, which represented the first habituation phase for the NOR testing. Animals were habituated to the room 60 min prior to the test. Testing

was performed under dim light (15 lux). The open field test box was cleaned with 70% ethanol in-between animals. Experiments were videotaped using a ceiling-mounted camera and were analyzed for time spent in the virtual center zone (33% of the total area) and total distance travelled using Ethovision 15.

3.1.4.8.4 Elevated Plus Maze

The Elevated Plus Maze test was used to assess anxiety-like behavior and was conducted as previously described (van de Wouw et al., 2020). Briefly, mice were allowed to explore the maze, which was elevated 1 m above the ground and consisted of a grey cross-shaped maze with two open arms and two closed arms (50 × 5 cm with 15 cm walls in the closed arms and 1 cm walls in the open arms) for five minutes. Mice were habituated to the room 30 min prior to the test. Experiments were conducted in red light (5 lux). The Elevated Plus Maze apparatus was cleaned with 70% ethanol in-between animals. Experiments were videotaped using a ceiling-mounted camera and time spent as well as the number of entries in the open arms, which was defined as all paws in the open arm, measured.

3.1.4.8.5 3-Chamber Social Interaction Test

Sociability and social novelty were assessed in a three-chamber apparatus as previously described (van de Wouw et al., 2020). The test consists of three sequential ten minutes' trials: (1) habituation; (2) sociability (analysis of time animals spent in the chamber with the conspecific mouse or with the object). (3) social novelty preference (analysis of time animals spent in the chamber with the novel mouse or in the chamber with the familiar mouse).

3.1.4.8.6 Morris Water Maze

The Morris Water Maze represents a test for spatial learning (Vorhees and Williams, 2006) and was conducted as previously described (Cryan et al., 2019). Briefly, mice

were trained over five days, with four trials per day each lasting two minutes to spatially allocate the submerged platform. On day six, the platform was removed and a probe trial lasting 30s conducted.

3.1.4.9 Plasma collection and corticosterone assay

Using Lithium-Heparin-coated capillaries, trunk blood was collected at the terminal time point of the study. Blood was centrifuged at 3500g at 4°C temperature for 15 min. Plasma was aspirated and stored at -80°C. Plasma CORT was measured as previously described(Scott et al., 2017) by ELISA, following vendor instructions (ENZO Corticosterone ELISA, cat. no. ADI-900-097). Concentration is expressed in ng/ml.

3.1.4.10 Cytokine assay

Cytokine secretion was assessed using the Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, cat. no. K15048D-1). Samples were run in duplicates. Reading and analyses were performed using the MESO QuickPlex SQ 120, SECTOR Imager 2400. Values under the fit curve range and detection range were excluded. Concentration is expressed in pg/ml.

3.1.4.11 Microglia analysis

For morphological characterization of microglia an Iba1 staining was performed with modifications as previously described(Boehme et al., 2014). Brains were sliced at -20°C in 30µm sections into antifreeze solution. Iba1 (ionized calcium-binding adapter molecule 1) is a Ca-dependent cytosolic protein that is expressed in the brain in microglia(Ito et al., 1998).

Iba1 staining was carried out using the 3,3'-Diaminobenzidine (DAB) method as described previously(Boehme et al., 2014). Briefly, slices (free-floating) were washed six times for 5 min in PBS, before sections were incubated for 30 min in 0.24% H₂O₂.

(in PBS) and then washed four times for 5 min each in PBS-T (PBS with 0.2% Triton). After blocking in PBS-T serum (0.2% Triton, 3% NDS) for 1 hour, incubation was carried out overnight at 4°C with the primary antibody (rabbit anti-Iba1, 1:2000 in PBS-T, #019-19741; Wako Pure Chemical Industries, Osaka, Japan). On day two, slices were washed four times for 5 minutes in PBS-T and blocked with 3% PBS-T serum for 15 minutes, followed by a two-hour incubation with the biotin-conjugated secondary antibody (Biotin-Sp conjugated donkey anti-rabbit IgG, 1: 500 in PBS-T; Jackson ImmunoResearch, cat. no. 711-065-152). After washing four times for 5 min in PBS-T, sections were transferred to Vectastain ABC peroxidase kit (Vector Laboratories, cat. no. PK-6100) for 1 h. Following a four-fold five-minute wash with PBS-T, the DAB reaction was started. Using the SIGMAFAST™ DAB Tablets (Sigma, cat. no. D4293), slices were put in DAB and the reaction started by adding 1:1 H₂O₂ urea, slices were subsequently incubated for ten minutes. After four times wash with PBS for five minutes, sections were mounted using DPX (Sigma, cat. no. 06522) as a mounting media and stored for analysis at room temperature.

The morphological complexity of microglia was assessed using the ImageJ-based "Sholl Analysis Tool"(Ferreira et al., 2014) and conducted following previously described guidelines(Gonzalez Ibanez et al., 2019). The focus was on the cornu Ammonis CA1 region of the hippocampus as it has been widely related to spatial memory(Tsien et al., 1996). The analysis was performed blinded. Altered morphology of the microglia, e.g. as a result of a lipopolysaccharide application, can be used to characterize whether the microglial cells are in the ramified or in the activated state. As a result of activation, microglial cells retract their processes into the cell body, thereby increasing its size(Kettenmann et al., 2011).

The Sholl analysis(Sholl, 1953) is a common technique used to evaluate and describe branching of cells, and is frequently used to analyze microglia morphology. The algorithm places concentric rings from a previously defined point and with a defined span and radius over the corresponding cell and counts the intersections of the

structures with these rings to automatically detect the number of branches. The "Advanced Sholl Analysis Tool" version 3.6.12 (<https://imagej.net/Sholl>) was used. Images were taken on the Olympus BX53 Upright Microscope with a 40x objective in Tagged Image File Format (.tif), to prevent losing any image information. The resolution was 2880x1800 pixels. The pictures were converted to 8-bit. Twelve cells per hemisphere per animal were evaluated. Cells were selected according to the following criteria: the entire cell had to be in focus, the cell had to be as clear as possible from its surrounding neighboring cells separable (i.e., the extensions should be as clearly as possible in the case of overlap), the cell was allowed on average not oblique be cut and it had to be always a single cell - duplications or superimpositions of several microglial cells in the area of Somas and/or the extensions were excluded. Care was taken that the analyzed cell within the frame was cropped according to the extent of branches that were clearly connected to the cell body of interest and fully separable from crossing branches of other cells. The evaluation of specifically selected microglial cells was performed as follows: First, the soma of the corresponding cell was marked using the ImageJ Polygon Selection tool. Thus, the parameters important for the further course could be grasped: the center of gravity of the cell (as starting point for the Sholl analysis), the area of the cell and the pixel coordinates, in order to cut out the soma from the later obtained skeleton. The cell with the highlighted soma was subsequently cut out of the corresponding image using the Freehand Selection tool. Then a binary image was created. This binary image was skeletonized in the next step. Subsequently, the soma was cut out. The focus point of the analyzed soma was marked and the Sholl analysis performed. The chosen radius was 2 to 60 μm to detect the complete cell. The distance of the concentric rings placed over each cell was 1 μm . On average, the first intersection was registered at 4 μm . Finally, the number of intersections per radius, the average maximum length of the projections and the soma size were evaluated per microglia.

3.1.4.12 Analysis of neurogenesis (BrdU+/NeuN+ staining, immunohistochemistry)

The same brains used in microglia analysis were used to assess the survival of newly born hippocampal neurons. Washing steps involve washing tissue in PBS on a gentle

plate mixer three times for 5 min each unless otherwise indicated. Free-floating sections were washed then incubated in 2N HCl at 37°C for 15 min. Sections were then rinsed twice in a sodium tetraborate buffer for 5 min, then washed in PBS. Blocking solution (10% normal donkey serum (NDS), 0.3% PBS-T) was added and sections set onto a gentle shaking plate for 1hr at RT. Sections were incubated in Anti-Rat BrdU (Abcam, cat. no. AB6326) in 1% NDS, 0.1% PBS-T, 1:100 dilution at 4°C overnight. Following BrdU antibody retrieval, sections were washed. All subsequent steps were carried out in darkness. Sections were incubated sections in secondary antibody (Donkey Anti-rat 594, 1:200; Thermo Fisher Scientific, cat. no. A-21209) for 90mins at RT in 1% NDS, 0.1% PBS-T in PBS, then washed. For NeuN staining, samples were blocked again for 30 min in the previously described blocking solution, then incubated in mouse anti-NeuN (Merck, cat. no. MAB377) in 1% normal goat serum (NGS; Abcam, cat. no. ab7481), 0.1% Triton-X 100, in PBS, 1:100 dilution at 4°C overnight. Sections were washed and placed in the second secondary antibody (Donkey Anti-mouse 488, 1:200; Thermo Fisher Scientific, cat. no. A-21202) for 90mins at RT in 1% NDS, 0.1% PBS-T. Finally, sections were washed, mounted onto slides and cover-slipped using PVB DABCO, and stored at 4°C until imaging. Stained slices were imaged under dim light at room temperature using an Olympus BX53 Upright Research Microscope. Hippocampal BrdU+/NeuN+ cells were quantified as cells that were distinguishably co-stained for both BrdU and NeuN fluorescent markers.

3.1.4.13 Intestinal motility assay

Gastrointestinal motility was assessed as previously described (Golubeva et al., 2017). Briefly, mice were single-housed at 8.00 a.m. with *ad libitum* access to food and drinking water. Three hours later, 0.2 ml of non-absorbable 6% carmine red in 0.5% methylcellulose dissolved in sterile PBS was administered by oral gavage, after which drinking water was removed. The latency for the excretion of the first red-colored fecal pellet was subsequently timed as a measure of gastrointestinal motility.

3.1.4.14 Assessment of fecal water content and weight

Mice were singly housed for 1 h during which fecal pellets were collected (five per animal). Pellets were subsequently weighed, dried at 50 °C for 24 h and weighed again. The average weight per pellet and percentage of fecal water content was calculated.

3.1.4.15 Telomere detection

Telomere attrition is a hallmark of aging (Lopez-Otin et al., 2013). To measure telomere length DNA was extracted from whole blood samples, which were collected at the end of the study, using the High Pure PCR Template Preparation Kit (Roche, cat. no. 11796828001) according to the manufacturer's instructions. Subsequently, the average telomere length was determined and calculated using the Absolute Mouse Telomere Length Quantification qPCR Assay Kit (ScienCell, cat. no. M8918) following the manufacturer's instructions.

3.1.4.16 Statistical analysis

Statistical analyses were conducted using SPSS 25 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 8.3.0 (GraphPad Software, Inc., La Jolla, CA, USA). Prior to statistical analysis, data were analyzed for normality using the Shapiro-Wilk test and for equality of variances using the Levene's test. Non-parametric data were analyzed by Kruskal-Wallis post-hoc Dunn's. Parametric data were analyzed using one-way analysis of variance (ANOVA) post-hoc Holm-Sidak, and is shown as Mean \pm SEM. All tests were two-sided where applicable. Outliers were excluded using the ROUT method (Motulsky and Brown, 2006) with Q = 1%. Statistical significance was set at $p < 0.05$.

3.2 Enduring neurobehavioral effects induced by microbiota depletion during the adolescent period

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3.2.1 Abstract

The gut microbiota is an essential regulator of many aspects of host physiology. Disruption of gut microbial communities affects gut-brain communication which ultimately can manifest as changes in brain function and behaviour. Transient changes in gut microbial composition can be induced by various intrinsic and extrinsic factors, however, it is possible that enduring shifts in the microbiota composition can be achieved by perturbation at a timepoint when the gut microbiota has not fully matured or is generally unstable, such as during early life or ageing. In this study we investigated the effects of 3-week microbiota depletion with antibiotic treatment during the adolescent period and in adulthood. Following a washout period to restore the gut microbiota, behavioural and molecular hallmarks of gut-brain communication were investigated. Our data revealed that transient microbiota depletion had long-lasting effects on microbiota composition and increased anxiety-like behaviour in mice exposed to antibiotic treatment during adolescence but not in adulthood. Similarly, gene expression in the amygdala was more severely affected in mice treated during adolescence. Taken together these data highlight the vulnerability of the gut microbiota during the critical adolescent period and the long-lasting impact manipulations of the microbiota can have on gene expression and behaviour in adulthood.

3.2.2 Introduction

The adolescent period is a key developmental period which marks the transition from childhood to adulthood (Paus et al., 2008). It is during this last developmental stage before adulthood that the brain is highly responsive to certain environmental cues that will shape neuronal architecture and promote maturation of social behaviours, emotional and cognitive capabilities and is hence a vulnerable period for the onset of psychiatric diseases (Borre et al., 2014). The gut microbiota composition of an adolescent is usually simpler and more unstable when compared with an adult, which is highly diverse and stable (Fouhy et al., 2012; Borre et al., 2014). These differences are probably due to relative immaturity of the gut microbiota during the adolescence, which make it more vulnerable to environmental stressors such as infection, use of antibiotic and poor diet. In addition to this, gonadal hormones are peaking during the puberty and it has been shown to have a long-term on the microbiota diversity (Paus et al., 2008; Yurkovetskiy et al., 2013). Overt changes in the gut microbiota composition might therefore contribute to the onset of such disease and could be targeted by the use of biotherapeutics, antibiotics or different types of diet to intervene (Borre et al., 2014; McVey Neufeld et al., 2016). Nonetheless, the consequences of gut microbiota manipulation during adolescence are yet to be fully understood.

The gastrointestinal tract is colonised by trillions of bacteria that are tightly associated with host physiology. When the equilibrium of the microbial milieu in the gut is shifted it can have long lasting effects on whole-body health including the brain and behaviour (Diaz Heijtz et al., 2011; Codagnone et al., 2018; Cryan et al., 2019). In mammals, the initial microbiota is obtained during the birthing process and develops alongside its host from a rather instable to a highly stable and diverse community in adulthood (Borre et al., 2014; Walsh et al., 2014). Microbiota composition during the developmental period is shaped by a combination of genetic and environmental factors to be highly adapted to the host and the host's environment (Walsh et al., 2014). However, maladaptation of the gut microbiota could affect innate and adaptative immunological players within the intestine and at site anatomically remote such as the brain and alter the host response to infection and vaccination as well as increase the susceptibility to brain disorders (Borre et al., 2014; Cowan et al.,

2019). For example, altered gut microbiota composition can immediately affect brain function by impacting the turnover and release of neurotransmitters, hormones as well as growth factors and consequently affect behavioural parameters and thereby increase the susceptibility to develop neuropsychiatric disorders (Verdu et al., 2006; Cryan and Dinan, 2012; Buffington et al., 2016; Leclercq et al., 2017).

One way to study the perturbation of the gut microbiota during critical periods is by using antibiotic-induced depletion. Antibiotics are one of the most important factors influencing the gut microbiota composition and structure (Hoban et al., 2016). Studies in both female and male mice (Cox et al., 2014; Hoban et al., 2016; Leclercq et al., 2017; Ruiz et al., 2017) and children (Ferrer et al., 2017; Korpela et al., 2018) have shown that antibiotic administration can induce changes in physiology, brain and behaviour. Antibiotic depletion of the microbiota for a defined time period represents an advantage in comparison to classic approach such as germ-free (GF) animals as the effects of microbiota depletion during the developmental period can be avoided (Luczynski et al., 2016; Lundberg et al., 2016). It has been shown, that the use of antibiotics to chronically deplete the gut microbiota during adulthood has been associated with hormonal changes and alterations in gene expression, decreased adult hippocampal neurogenesis, and changes anxiety-related responses, exploratory behaviour, and cognitive abilities (Desbonnet et al., 2015; Frohlich et al., 2016; Hoban et al., 2016). Moreover, early life exposure to antibiotics induces long-lasting increases in visceral pain responses (O'mahony et al., 2014) as well as altered metabolic programming (Cox et al., 2014). We have previously shown that microbiota depletion with antibiotics commencing in adolescence all the way through adulthood resulted in deficits in anxiety and cognitive behaviour (Desbonnet et al., 2015). It is not clear whether such changes are a result of microbiota changes specifically in adolescence, in adulthood or a combination.

Thus, in these experiments we investigated the consequences of gut microbiota depletion specifically during adolescence or adulthood and their associated long-term effects on emotional and cognitive behaviours and related-neurochemical measures.

3.2.3 Experimental Procedures

3.2.3.1 Animals and experimental design

Adolescent and adult male C57Bl/6OlaHsd mice (Envigo, UK) were housed 4 per cage in standard cages. All mice were housed in our animal facility and maintained under a 12-h light/dark cycle. All experiments were conducted in accordance with the European Directive 86/609/EEC. Approval by the Animal Experimentation Ethics Committee of University College Cork and Health Products Regulatory Authority were obtained before commencement of all experiments. To comply with 3Rs (reduction, refinement and replacement) and animal welfare, the adolescent aspect of the experiment was run simultaneously with another experiment investigating the gut microbiota (Fülling et al., 2020). The same control group was used in this study.

In order to sufficiently deplete the gut microbiota, a wide-spectrum antibiotic cocktail (ABX) consisting of ampicillin (1 g/L, CAS no. 69-52-3), vancomycin (0.5 g/L, CAS no. 1404-93-9), ciprofloxacin HCL (0.2 g/L, CAS no. 93107-08-5), imipenem (0.25 g/L, CAS no. 74431-23-5) and metronidazole (1 g/L, CAS no. 443-48-1) was prepared (Fig. 1B) (Hoban et al., 2016; Fülling et al., 2020). All substances were purchased from Discovery Fine Chemicals, UK. This antibiotic cocktail has little to no oral bioavailability and was prepared freshly with autoclaved water every second day for three weeks (Frohlich et al., 2016). Control mice received autoclaved water (CTRL). Mice were treated during adolescence (P28-P49) or adulthood (P76-97) and will be referred to as ABX_{adolescence} and ABX_{adulthood}, respectively (see 3.2-Fig. 1A). Behavioural tests commenced 24 days after the final antibiotic exposure. Mice were equally assigned to experimental groups based on bodyweight to ensure equally distribution among the groups. Behavioural tests investigating aspects of anxiety, cognition, social behaviour and fear conditioning were chosen as these behaviours have been shown to be affected by alterations in gut microbiota composition and structure [14, 41–44]. Tissue samples were collected 24 h after the last behavioural test. Body weight was monitored throughout the experiment. The investigators who were involved in sample processing and data analysis were blinded to the groups. All behaviours were assessed by two independent scorers blind to the groups. See

supplemental methods for detailed information of all procedures and analysis run in this study.

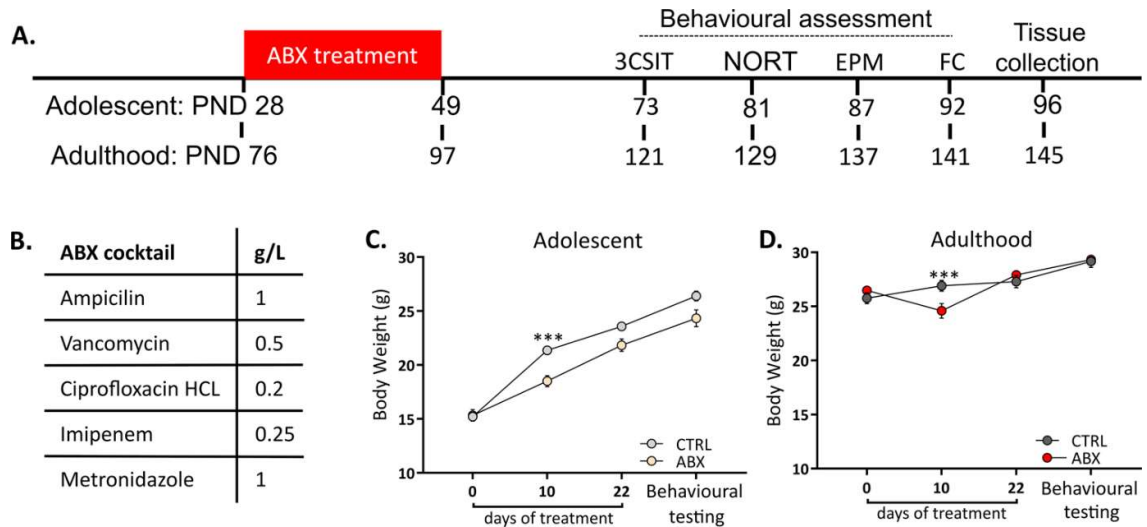


Fig. 3.1-1: Experimental design and body weight performance during the experiment. A Schematic representation of the experimental timeline. Numbers represent the age of the mice at that specific timepoint. B List of drugs used for the antibiotic cocktail. C, D Changes in body weight during adolescence (C) and adulthood (D) over the time course of the experiment. ABX-treated mice show a significant body weight loss on PND10 which is restored afterwards. Mean \pm SEM. * $p < 0.05$. Sample size for adolescence: CTRL $n = 12$ and ABX $n = 10$, adults: CTRL and ABX $n = 11$. Two-way repeated measures ANOVA followed by Sidak's post hoc test. PND: postnatal day, CTRL: control, ABX: antibiotic, 3CSIT: three-chambered social interaction test, NORT: novel object recognition task, EPM: elevated plus maze, FC: fear conditioning, Beh: behavioural test.

3.2.3.2 Elevated Plus Maze

The elevated plus maze (EPM) was used to investigate anxiety-like behaviours (Fülling et al., 2020). Mice were allowed to explore the maze for 5 min; the time spent in the open arms, as well as the number of entries into the arms and head dips were analysed.

3.2.3.3 Novel Object Recognition Task

Novel object recognition task (NORT) is a test for working memory (Desbonnet et al., 2015). Mice were exposed to two identical objects which they could explore for 10 min. One hour later they were exposed to a familiar and a new object. The time they spent exploring the new object was taken as indication of their memory function.

3.2.3.4 Three-chamber social approach test

Sociability and social novelty were investigated using the three-chamber social interaction test (3CSIT) (Fülling et al., 2020). The test consisted of three sequential 10-min trials: (1) habituation, (2) sociability, measured as the time the mouse spent in proximity to a conspecific or an object and (3) social novelty preference as measured by the time the mouse spent with an unfamiliar conspecific or a familiar one.

3.2.3.5 Differential fear-conditioning paradigm.

Fear conditioning (FC) is based on pairing an initially neutral and non-aversive stimulus, an auditory cue or context (conditioned stimulus, CS), with an aversive stimulus, such as a foot shock (unconditioned stimulus, US), which will result in a fear response in the presence of the CS (Verma et al., 2015). The paradigm was run over four consecutive days: day 1 (context A): conditioning, day 2 (context A): contextual extinction, day 3 (context B): conditioned extinction in a novel context, day 4 (context B): context recall.

3.2.3.6 RNA extractions, reverse transcription and quantitative RT-PCR

Whole amygdala and prefrontal cortex were rapidly gross-dissected on an ice-cooled Petri dish following coordinates described in the “The Mouse Brain in Stereotaxic Coordinates” (Paxinos and Franklin, 2012) and snap-frozen on dry-ice. These brain regions were chosen as they are major contributor to anxiety and fear learning

(amygdala) and proper neuronal communication during development (prefrontal cortex) (Bravo et al., 2011; Stilling et al., 2015; Hoban et al., 2016; Cowan et al., 2018; Stilling et al., 2018). The list of genes was elaborated together with the experimental design to assess major components modulated by the gut microbiota, such as immune and microglia-related markers, tight junction proteins and myelin- and stress-related genes (Stilling et al., 2015; Hoban et al., 2016; Stilling et al., 2018; Cryan et al., 2019; Fülling et al., 2020). Additionally, based on the behavioural phenotype, we included genes associated with anxiety-like behaviour such as the genes involved in the GABAergic and glutamatergic system, NPY system and genes related with neuroplasticity (Bravo et al., 2011; Frohlich et al., 2016; Lach et al., 2018; Cryan et al., 2019; Fülling et al., 2020). Total RNA was extracted with the mirVana total RNA extraction kit (Ambion, UK) and RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, USA) in a G-storm thermocycler (G-storm, Surrey, UK). Real-time PCR was performed on the cDNA samples using SYBR green (SensiFAST™ SYBR®, BioLine, UK) and gene expression levels were analysed on an AB7300 system (Applied Biosystems, Thermo Fisher Scientific, USA). Expression levels were calculated as the average of three replicates for each biological sample from all three groups relative to the endogenous control. Fold changes were calculated using the $\Delta\Delta C_t$ method (Fülling et al., 2020). The expression of the housekeeper ACTB was not affected by ABX treatment or age.

3.2.3.7 Caecal microbiota composition (16S rRNA gene sequencing)

The QIAmp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) was used for caecal DNA extraction. The procedure was coupled with an initial bead-beating step. Amplification and preparation for sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was done as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol and as previously described (Fülling et al., 2020). Briefly, microbial genomic DNA was run with each primer (forward primer (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3').

PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, UK). Dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, USA). PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, USA). Following, samples were run on the Agilent Bioanalyser for quality analysis and samples prepared for sequencing following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform (Clinical Microbiomics, Denmark).

3.2.3.8 Microbiome bioinformatics processing

Three hundred base pair paired-end reads were prefiltered based on a quality score threshold of >28 and trimmed, filtered for quality and chimerae using the DADA2 library in R (Callahan et al., 2016). Samples with fewer than 10,000 reads after trimming and filtering were dropped. Taxonomy was assigned with DADA2 against the SILVA SSURF database release v132. Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. ASVs that were only detected as non-zero in 2 or fewer of total samples were excluded.

3.2.3.9 Statistics

Power analysis was performed beforehand using the Software G*Power 3.1 to ensure adequate sample size number to detect changes in behaviour and gene expression. Statistical analysis and plotting were conducted using Prism 7 (GraphPad, USA). Data were checked for normality using D'Agostino & Pearson normality test and the ROUT method was used to check for outliers. Two-tailed Welch's t-test was used for comparison between ABX treated mice and their respective controls for the EPM, NORT as well as context and extinction recall in FC. Two-way analysis of variance (ANOVA) was used for 3CSIT and two-way repeated measures ANOVA was used to analyse data for body weight as well as acquisition and cued extinction in FC. Sidak's multiple comparisons post hoc test was used where applicable. Statistical significance was set at $p < 0.05$.

Statistical analysis of microbiota data was performed using the R software (version 3.6) environment with Rstudio (version 1.1.453). Alpha diversity was calculated using the iNEXT library (Hsieh et al., 2016). Wilcoxon Rank Sum followed by Bonferroni post hoc tests were used to assess differences in Alpha diversity scores. For principal component analysis (PCA), permutational multivariate analysis of variance (PERMANOVA) was used to identify relationships of significance between variables using the `adonis()` function from the `vegan` library on Aitchison distance matrices calculated with the ALDEx2 library (Fernandes et al., 2014). A pairwise implementation of the ALDEx2 function `aldex.t.test()` was also used to calculate pairwise differential abundance, using the Bonferroni procedure as a post-hoc. In the case of microbiome analysis, the benjamini-hochberg procedure was used to account for false discovery rate due to multiple comparisons, a q-value of 0.1 was deemed significant.

3.2.4 Results

3.2.4.1 Antibiotic treatment in adolescence affects body weight

Two-way repeated measures ANOVA showed an overall effect of ABX treatment during adolescence on body weight (interaction $F(3,48)=3.84$, $p=0.015$, treatment $F(1,16)=7.18$, $p=0.017$), whereas such an effect was not seen following treatment with $ABX_{adulthood}$ (interaction $F(3,60)=12.56$, $p<0.001$, treatment $F(1,20)=0.11$, $p=0.75$; Fig. 3.2-1C, D). Sidak's multiple comparison, however, revealed that body weight was significantly lower in the ABX treatment in both $ABX_{adolescence}$ and $ABX_{adulthood}$ mice on day 10 of the treatment ($p=0.003$ and $p=0.05$, respectively).

3.2.4.2 Microbial diversity was only affected by ABX treatment during adolescence

To investigate possible long-lasting effects of ABX treatment during adolescence and adulthood on the gut microbiota, microbiota was sequenced from caecal contents 49 days after termination of the treatment. Overall, sequencing demonstrated that microbial diversity and structure was shifted only in $ABX_{adolescence}$ mice (Fig. 3.2-2A-

C). With regards to beta-diversity PERMANOVA identified significant differences between ABX_{adolescence} mice when compared to their controls ($p < 0.001$, $R^2 = 0.193$, $p_{adj} = 0.004$; Fig. 3.2-2A), but no such change was observed in ABX_{adulthood} ($p = 0.029$, $R^2 = 0.071$, $p_{adj} = 0.174$; Fig. 3.2-2A).

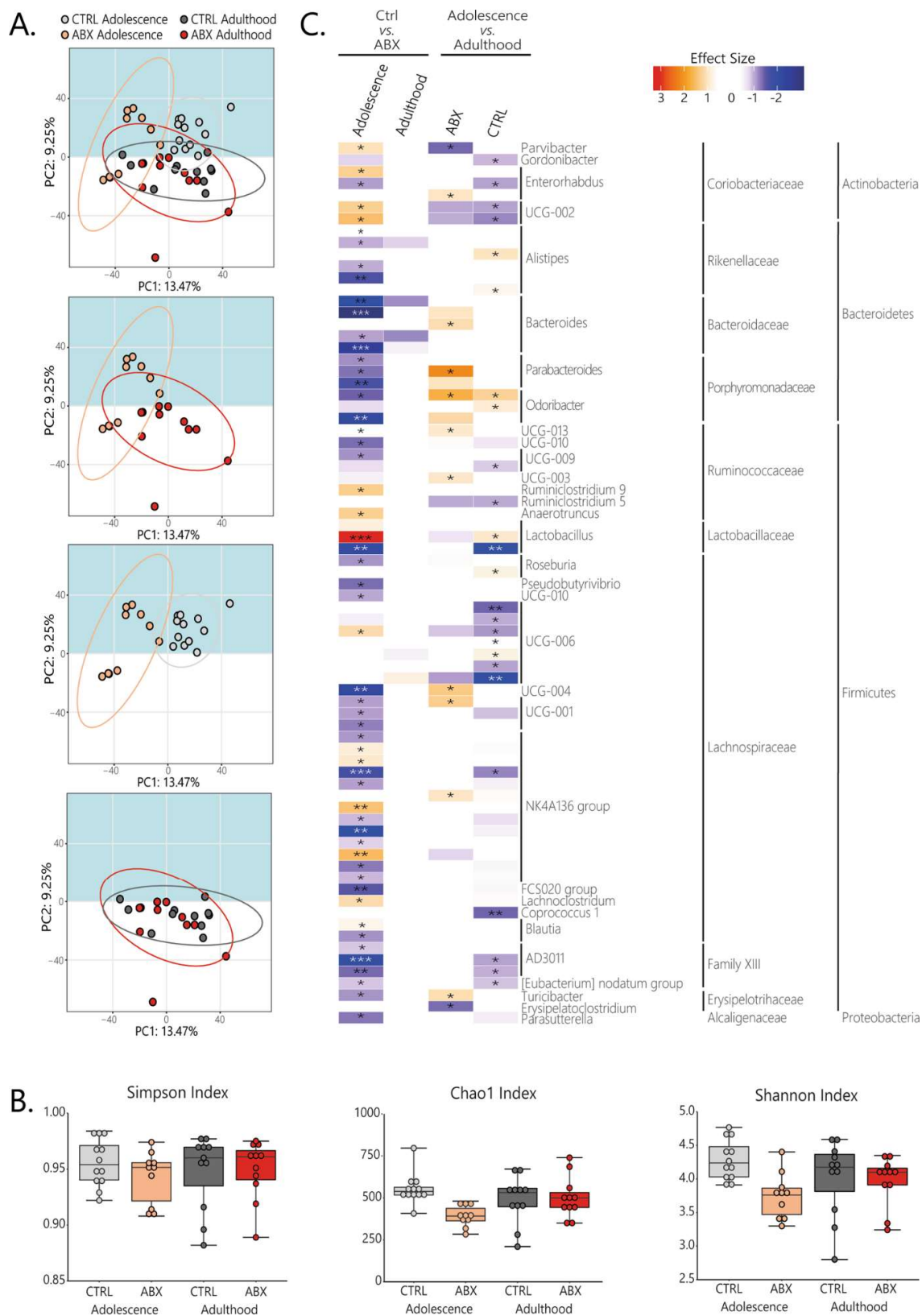


Fig. 3.2-2: Long-lasting changes in caecal microbiota composition following ABX intervention during adolescence. A PCA blots depicting differences in beta-diversity

*between mice treated with ABX and their respective controls. While beta-diversity was affected by ABX_{adolescence} treatment no such effects were seen following ABX_{adulthood} treatment. B alpha-diversity indices: Chao1, Shannon and Simpson. Changes in the Chao1 and Simpson indices are observed following treatment with ABX during adolescence but not after treatment in adulthood. C Heat map representing relative abundance of ASVs. Significant differences were observed between control mice and ABX-treated mice during adolescence but not when treated during adulthood. Some differences in ASVs were observed when comparing both ABX treatments and controls with one another. The latter does not explain the effect on relative abundance of ABX treatment during adolescence. Mean \pm SEM. * $p < 0.05$. Sample size for adolescence: CTRL $n = 12$ and ABX $n = 10$; for adults: CTRL and ABX $n = 11$. A Permutational multivariate analysis of variance (PERMANOVA), followed by pairwise PERMANOVA post hoc Benjamini–Hochberg, B Wilcoxon rank-sum test, post hoc Bonferroni, * $p < 0.05$, C Mann–Whitney U test post hoc Benjamini–Hochberg, Benjamini–Hochberg false discovery rate (FDR) $q < 0.2$. Asterisks in the heat map represent the following q values: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.*

Alpha diversity, which describes the diversity of species within a community, was measured by calculating indices for richness and evenness based on amplicon sequence variants (ASV) level. Figure 3.2-2B shows reduced numbers of bacterial species between ABX_{adolescence} and their controls (Chao1 Index; pairwise comparison using Wilcoxon rank sum test, $p < 0.001$) while no changes were observed in ABX_{adulthood} mice. Moreover, a significant decrease in evenness across ASVs was observed in ABX_{adolescence} mice (Shannon Index; pairwise comparison using Wilcoxon rank sum test, $p < 0.001$), indicating that treatment with ABX during adolescence disrupted the uniformity of the population size of each of the species present. No effects of treatment during adolescence or adulthood were observed for the Simpson's index.

ABX treatment only resulted in significant changes in relative abundance comparing ABX treated mice with controls when mice were exposed to ABX during the adolescent period. In comparison to control mice, ABX_{adolescence} mice showed reduced relative abundance of most of the strains affected. At the phylum level, Firmicutes and Bacteroidetes were most affected by the ABX_{adolescence} intervention (Fig. 3.2-2C). Changes in the phylum Actinobacteria were restricted to the family *Coriobacteriaceae*, with increased numbers in the genus UCG002 while the genus *Enterorhabdus* was differentially affected depending on the ASVs. Relative

abundance of all the affected genera in the phylum Bacteroidetes was decreased, with the strongest reduction in the genera *Bacteroides*. Firmicutes was the phylum most affected by ABX_{adolescence} treatment. Genera in this phylum were differentially affected by ABX_{adolescence} treatment. In the family *Lachnospiraceae* relative abundance of all affected ASV of the genera *Rosburia*, *UCG-001*, *UCG-004*, *UCG-010*, *FCS020* group and *Pseudobutyrvibrio* was decreased, whereas at least one ASV from the genera *UCG-006*, *NK4A136* group, *Lachnoclostridium* and *Blautia* showed increased relative abundance following ABX_{adolescence} treatment. Of the affected ASVs of the family *Ruminococcaceae* relative abundance of all the ASVs in the genera *UCG-003*, *UCG-009*, *UCG-010* and *UCG-013* was decreased while one ASV each of the genera *Ruminiclostridium 5*, *Ruminiclostridium 9* and *Anaerotruncus* was significantly increased. Similarly, one ASV of the genus *Lactobacillus* of the family *Lactobacillaceae* was strongly decreased, whereas another ASV of this genus was strongly increased following ABX_{adolescence} treatment. The relative abundance of all affected ASVs of the family *Family XIII* and *Erysipelotrichaceae* was decreased. The only affected ASV of the phylum Proteobacteria was also decreased. No significant effects of ABX_{adulthood} on relative abundance were observed. However, differences in relative abundance were observed when comparing ABX treated groups or control groups with one another. When comparing ABX treated groups, relative abundance of most of the ASVs is increased in ABX_{adulthood} mice in comparison to ABX_{adolescence} treated mice and vice versa (e.g. ASVs of the genera *Parabacteroides* and *Odoribacter*). This is in line with the decrease observed in the comparison of relative abundance of ABX_{adolescence} mice and their controls. Similarly, the differences seen between the controls of either treatment is not in contradiction with the differences observed in relative abundance of ABX_{adolescence} mice in comparison to their controls. The differences in relative abundance between controls mostly occurred in ASVs that are not affected in the ABX_{adolescence}, 14 out of the 25 ASVs that were different between the controls were not significantly affected by ABX_{adolescence} treatment (e.g. ASVs of the genus *Alistipedis*).

3.2.4.3 Treatment with antibiotics strongly affected anxiety-like behaviour, with a more pronounced effect in mice treated during adolescence

To test effects of ABX treatment on anxiety-like behaviour, mice were tested in the EPM. Mice that underwent antibiotic treatment showed increased anxiety-like behaviour. Unpaired t-test with Welch's correction showed that the percentage of time into the open arms as well as the number of head dips ($t=2.846$, $p=0.01$ and $t=2.909$, $p=0.01$, respectively; Fig. 3.2-3A, B) were affected significantly in ABX_{adolescence} mice. Student t-test showed that the percentage of entries into the open arms also was affected significantly in ABX_{adolescence} mice ($t=2.529$, $p=0.02$; Fig. 3.2-3C). Welch's t-test revealed no statistical significance in the percentage of entries into the open arm in ABX_{adulthood} mice ($t=1.946$, $p=0.06$; Fig. 3.2-3K). Mann-Whitney non-parametric test found that the percentage of time into the open arms as well as head dips were no difference in ABX_{adulthood} compared to the controls ($U=38$, $p=0.148$ and $U=38$, $p=0.145$, respectively; Fig. 3.2-3I, J). Entries into the closed arm did not differ between treatment groups (adolescence: $t=0.419$, $p=0.68$; adulthood: $t=0.449$, $p=0.65$; Fig. 3.2-3D, L), indicating that ABX treatment did not affect locomotor activity.

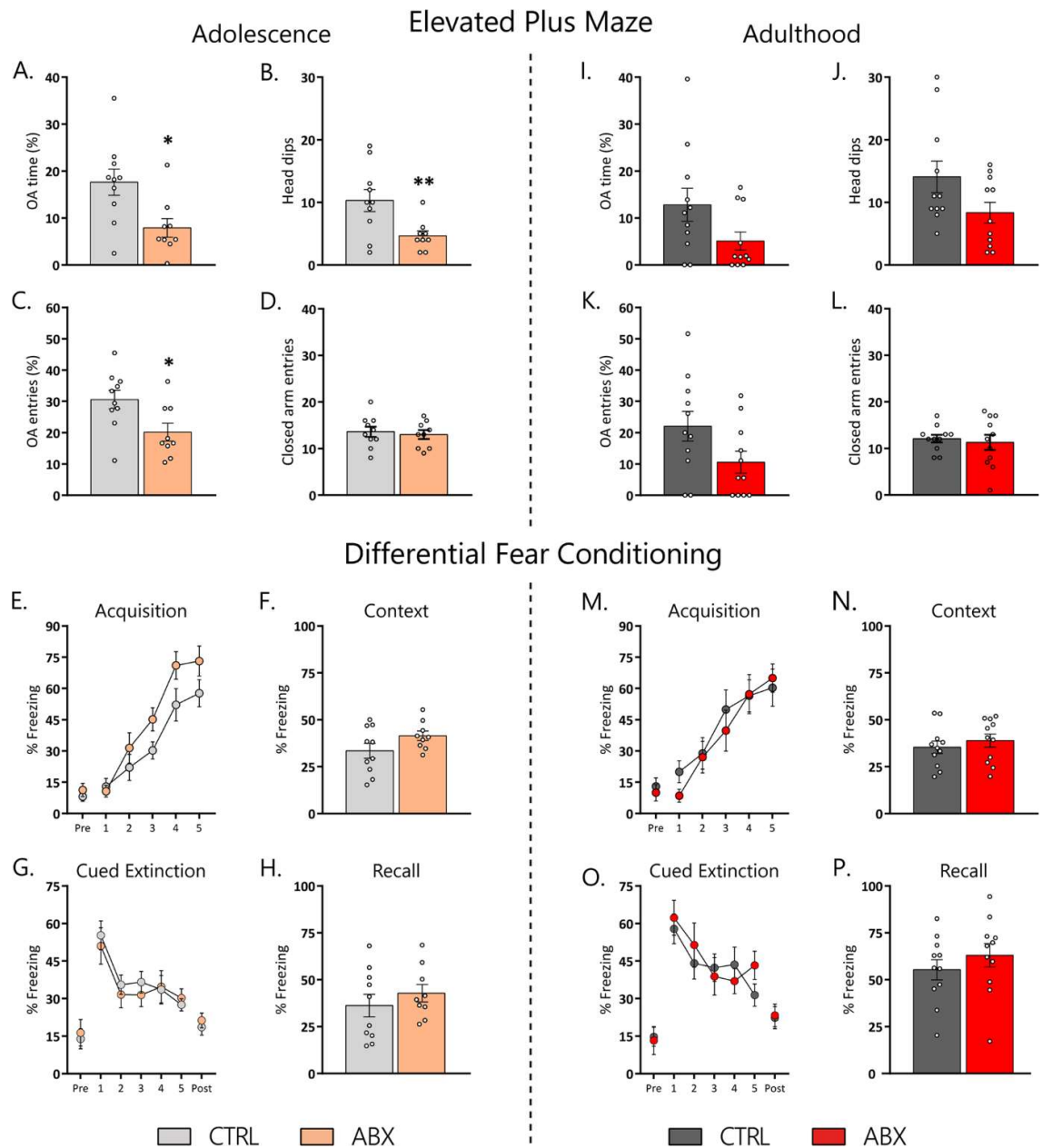


Fig. 3.2-3: ABX treatment during adolescence affects anxiety-like behaviour and the fear response during fear acquisition. A–D Mice treated with ABX during adolescence showed increased anxiety-like behaviour as they showed decreased % time in and % entries into the open arm as well as decreased number of head dips. I–L No significant decrease in anxiety-like behaviour was seen in mice treated with ABX during adulthood. E–H An effect of treatment was seen for the acquisition of fear conditioning in mice treated with ABX during adolescence, but no other parameter was affected. M–P Treatment with ABX during adulthood had no effect on any parameter measured in the fear-conditioning paradigm. Mean \pm SEM. A–D, F, H, I–L Welch's *t*-test comparison between CTRL and ABX. E, G, M, O two-way repeated measures ANOVA, followed by Sidak's post hoc test when applicable **p* < 0.05 and

***p < 0.01. Sample size for adolescence (CTRL: n = 9; ABX: n = 10) and adulthood (CTRL: n = 11; ABX: n = 11).*

3.2.4.4 Sociability and memory processes were not affected by antibiotic treatment in adolescence or adulthood

As social behaviour and cognition have been shown to be impacted by alterations in gut microbiota composition, we investigated the effects of ABX treatment on measures in 3-CSIT and NORT. ABX treatment during adolescence or adulthood had no effects on sociability or social novelty. Control and ABX treated mice both preferred a stranger mouse (S1) over an innate object (O) (two-way repeated measures ANOVA, interaction: S1 x O: $F(1,17)=52.92$, $p<0.001$, treatment: $F(1,17)=1.76$, $p=0.20$ for adolescence; interaction: S1 x O: $F(1,20)=0.59$, $p=0.44$, treatment: $F(1,20)=0.013$, $p=0.90$ for adulthood, supplementary Fig. 1B, E, respectively). Similarly, no effect of ABX treatment was seen on social novelty, as no differences were observed between ABX treated mice and their respective controls (two-way repeated measures ANOVA, interaction: S1 x S2: $F(1,17)=13.96$, $p<0.01$, treatment: $F(1,17)=3.03$, $p=0.09$ for adolescence; interaction: S1 x S2: $F(1,20)=2.74$, $p=0.11$, treatment: $F(1,20)=0.61$, $p=0.44$ for adulthood, supplementary Fig. 1C, F, respectively)

In the NORT, neither ABX_{adolescence} or ABX_{adulthood} exhibited differences in the ability to recognize a distinct object one hour after the training session, expressed by the time exploring the novel object (adolescence: $t=1.233$ $df=16.47$, $p=0.23$; adulthood: $t=0.5288$ $df=16.77$, $p=0.60$, supplementary figure 2A, D).

3.2.4.5 Fear learning is only affected in animals treated with antibiotics during adolescence

Fear conditioning aimed to investigate different fear responses, such as fear learning, contextual fear, and cued extinction (Fig. 3.2-3E-H, M-P). All experimental groups learned the task as indicated by increasing freezing behaviour during acquisition (two-way repeated measures ANOVA; CS presentation, adolescence: $F(4,68)=38.11$, $p<0.001$; adulthood: $F(4,80)=27.36$, $p<0.001$; Fig. 3.2-3E, M). Only ABX_{adolescence} mice significantly increased freezing behaviour compared to the control group during fear

acquisition, while in adults, ABX did not affect fear acquisition (two-way repeated measures ANOVA; treatment, adolescence: $F(1,17)=4.48$, $p=0.04$; adulthood: $F(1,20)=0.17$, $p=0.67$). Sidak's multiple comparison post hoc test did not find a significance between CTRL and ABX in any timepoint in both adolescence and adults mice. On day 2, contextual fear was assessed. Unpaired t-test with Welch's correction revealed that the treatment did not affect freezing behaviour in adolescent mice ($t=1.70$, $p=0.10$; Fig. 3.2-3F). Student t-test revealed the same in adult mice ($t=0.73$, $p=0.47$; Fig. 3.2-3N). In the cued extinction session (day 3), the response to the CS (paired sound) was analysed. All experimental groups reduced their freezing response over the course of CS presentation (CS presentation, two-way repeated measures ANOVA adolescence: $F(4,68)=18.03$, $p<0.001$, adulthood: $F(4,80)=15.15$, $p<0.001$; Fig. 3.2-3G, O), suggesting that all experimental groups were able to extinguish cued fear. Fear extinction was not affected by treatment (two-way repeated measures ANOVA; adolescence: $F(1,17)=0.095$, $p=0.76$, adulthood: $F(1,20)=0.11$, $p=0.74$). Pre and post CS freezing were similar in ABX treated mice compared to their controls (Welch's t-test; adolescence; pre: $t=0.36$, $P=0.72$; post: $t=0.61$, $p=0.54$; adulthood; pre: $t=0.22$, $p=0.82$; post: $t=0.15$, $p=0.88$), indicating the absence of generalized fear. Fear recall was tested on day four and no difference was observed (Welch's t-test; adolescence: $t=0.86$, $p=0.39$, adulthood $t=0.93$, $p=0.36$; Fig. 3.2-3H, P). Taken together, this data show that transient depletion of the gut microbiota has little potential to long-lasting affect fear response.

3.2.4.6 Amygdala gene expression is more strongly affected by treatment during adolescence than adulthood

Many studies have demonstrated a link between the gut microbiota and gene expression in the amygdala [52, 58]. In amygdala homogenates, mRNA levels revealed a stronger effect of ABX_{adolescence} than ABX_{adulthood} treatment (Fig. 3.2-4 and Table 1). The immune response was heavily affected by ABX treatment during adolescence and adulthood (Fig. 3.2-4A, E). For instance, elevated levels of *Fcgr2b* (Fc Fragment of IgG Receptor IIb) gene were observed in both ABX_{adolescence} and ABX_{adulthood} mice. The toll-like receptor 4 gene (*Tlr4*) was oppositely affected by ABX

treatment with elevated gene expression following ABX_{adolescence} treatment and reduced expression after ABX_{adulthood} treatment. In contrast, interleukin 1b (*Il1b*) was elevated following ABX_{adulthood} treatment but not following ABX_{adolescence} treatment, while the microglia-related gene *C1qa* (encodes the A-chain polypeptide of serum complement subcomponent C1q) and *Cx3cr1* (interleukin 8 receptor, alpha) were only upregulated in ABX_{adolescence} mice. *Rac2* (Rho GTPase Rac2), which regulates phagocytosis was upregulated in both experimental groups. The complement component 3 (*C3*) which is also involved in the regulation of phagocytosis, was only upregulated in ABX_{adulthood} mice. Classical and peptidergic neurotransmission was strongly affected by ABX_{adolescence} treatment but remained mostly unaltered in ABX_{adulthood} mice (Fig. 3.2-4B, F). The levels of gamma-aminobutyric acid receptor subunit alpha-2a (*Gabra2*) and beta-1b (*Gabrb1*), the metabotropic glutamate receptor subunit 5 (*Grm5*), neuropeptide Y (NPY) and its Y1 receptor (*Npy1r*) and the glucocorticoid receptor (*Nr3c1*) were only increased in ABX_{adolescence} mice. The corticotropin-releasing hormone receptor subunit 1 (*Crh1r*) was the only gene affected in ABX_{adulthood} mice, that was not altered by ABX_{adolescence} treatment. Neuroplasticity genes were differently affected by ABX_{adolescence} and ABX_{adulthood} (Fig. 3.2-4C, G). While the levels of postsynaptic density protein 95 (*Psd95*) and synaptophysin (*Syp*) were increased in ABX_{adolescence} mice, these genes were downregulated following ABX_{adulthood} treatment. Interestingly, genes associated with short chain fatty acids (SCFAs), the solute carrier subtypes (*Slc5a8* and *Slc16a1*) were only elevated in ABX_{adolescence} mice. Similarly, the gene expression of tight junction genes, such as occludin (*Ocln*) and tight-junction protein 1 (*Tjp1*) were only increased in ABX_{adolescence} mice. Taken together, although more genes are affected in ABX_{adolescence}, immune-related genes are similarly affected by both ABX treatments.

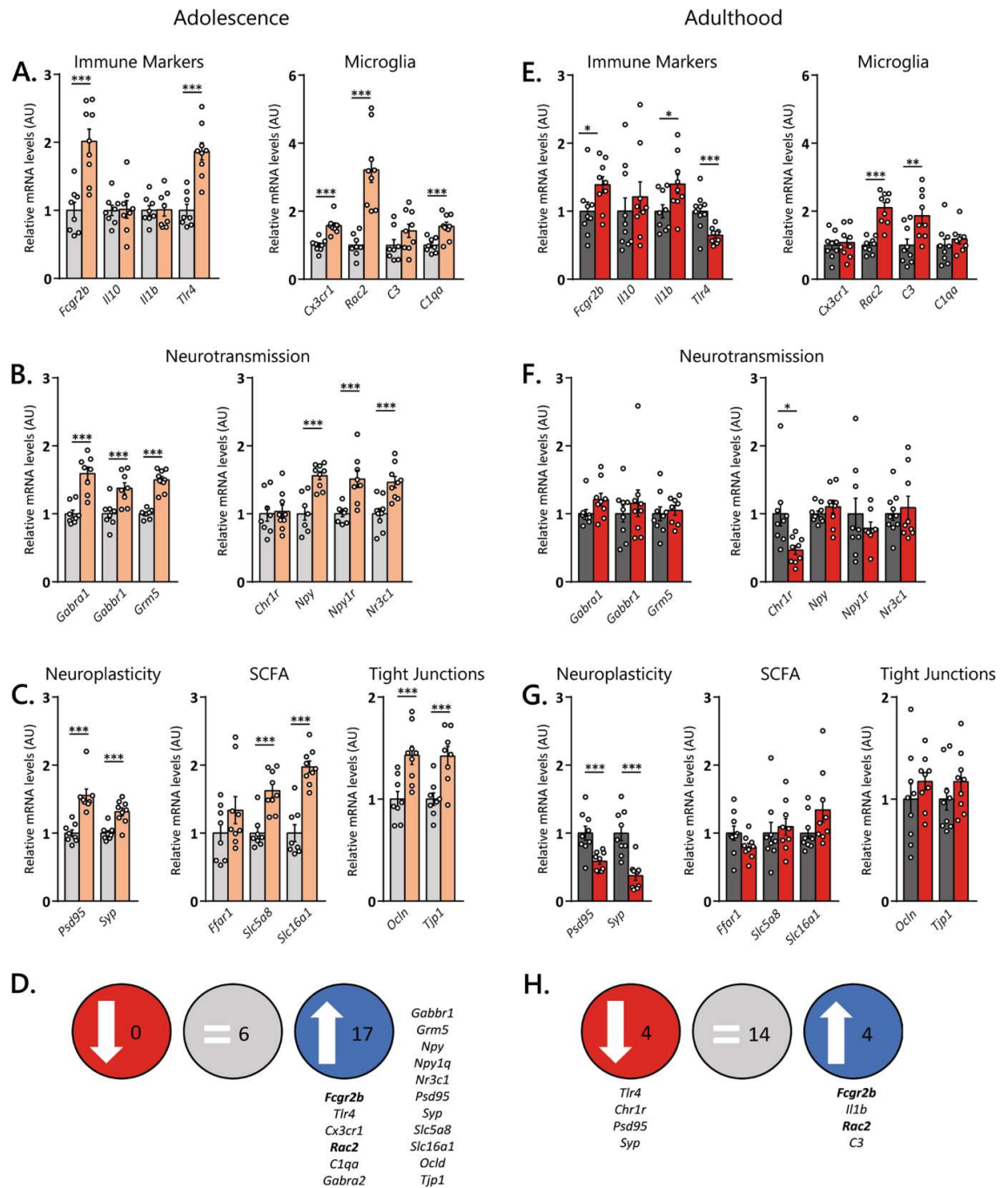


Fig. 3.2-4: Amygdalar gene expression is more drastically changed following ABX treatment during adolescence. A, B While genes related to neuroimmunity and microglia are similarly affected in ABXadolescence and ABXadulthood mice, gene expression genes involved in C, D neurotransmission, E, F neuroplasticity, short-chain fatty acids and tight-junction proteins are more drastically affected in ABXadolescence mice. Mean \pm SEM. Unpaired *t*-test with Welch's correction comparing vehicle and antibiotic treatment for each gene. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001. Sample size for adolescence: CTRL and ABX *n* = 8 (Il10, Il1b, Gabra2, Gabbr1, Npy1r, Psd95 and Tjp1), CTRL *n* = 8 and ABX *n* = 9 (Fcgr2b, Cx3cr1, Rac2, C3,

C1qa, Grm5, Chr1r, Npy, Syp, Ffar1, Slc5a8, Slc16a1, Ocln), CTRL *n* = 9 and ABX *n* = 8 (*Tlr4*), CTRL and ABX *n* = 9 (*Nr3c1*). Sample size for adults: CTRL and ABX *n* = 9 (*Fcgr2b, Il1b, Cx3cr1, Rac2, C3, Gabra2, Gabbr1, Grm5, Crh1r, Npy1r, Psd95, Syp, Ffar1, Slc5a8, Ocln, Tjp1*), CTRL *n* = 10 and ABX *n* = 9 (*Il10, Tlr4, Nr3c1, Slc16a1*), CTRL *n* = 9 and ABX *n* = 8 (*C1qa, Npy*). SCFA: short-chain fatty acid, *C1qa*: complement C1q subunit A, *C3*: complement component 3, *Crh1r*: corticotropin-releasing hormone receptor 1, *Cx3cr1*: chemokine receptor 1, *Fcgr2b*: Fc fragment of IgG receptor IIb, *Ffar1*: free fatty acid receptor 1, *Gabrb1*: gamma-aminobutyric acid type B receptor subunit 1, *Gabra2*: gamma-aminobutyric acid type A receptor alpha2 subunit, *Grm5*: glutamate metabotropic receptor 5, *Il1b*: interleukin 1b, *Il10*: interleukin 10, *Npy*: neuropeptide Y, *Npy1r*: neuropeptide Y receptor Y1, *Nr3c1*: glucocorticoid receptor, *Ocln*: occludin, *PSD95*: postsynaptic density protein 95, *Rac2*: Ras-related C3 botulinum toxin substrate 2, *Slc5a8*: solute carrier family 5 member 8, *Slc16a1*: solute carrier family 16 member 1, *Syp*: Synaptophysin, *Tjp1*: tight-junction protein 1, *Tlr4*: toll-like receptor 4.

Finally, we analysed myelination-related genes in the prefrontal cortex, which have been suggested to be modulated by the gut microbiota [38, 59]. Therefore, we analysed gene expression of myelin regulatory factor (*Myrf*), proteolipid protein 1 (*Plp1*) and the transcription factor SOX-10 (*Sox10*). ABX_{adulthood} treatment resulted in increased gene expression of *Myrf* and *Sox10* while ABX_{adolescence} showed no effects (Supplementary Fig. 2A-B).

3.2.5 Discussion

Adolescence is a particularly vulnerable time for the onset of psychopathology. Understanding what factors mediate such susceptibility is an important area in biological psychiatry research. In the present studies we assessed the long-term effects of transient gut microbiota depletion using an ABX cocktail in mice during adolescence or adulthood. ABX_{adolescence} had long-lasting effects on composition and structure of the gut microbiota and anxiety-like behaviour, whereas ABX_{adulthood} had no effects. This long-lasting shift in the gut microbiota when depleted during adolescence highlights the vulnerability of the gut microbiota during development and may be responsible for the anxiety-like phenotype and pronounced changes in gene expression within the amygdala observed in ABX_{adolescence} mice. To our knowledge, this is the first study demonstrating that the long-lasting effects on gut microbiota only occur when treated during a critical developmental period.

In mammals, the initial microbiota is acquired during the birthing process and is shaped during the developmental period by genetics and environmental factors until early adulthood (Borre et al., 2014; Ruiz et al., 2017; Korpela et al., 2018). During this developmental process, the gut microbiota adapts to its host's needs and becomes increasingly stable and resistant to challenges such as ABX treatment or transient changes in diet. Here, we observed long-lasting changes in alpha- and beta-diversity as well as relative abundance of the gut microbiota following exposure to ABX_{adolescence} treatment only. ABX intervention during early development is characterized by long-lasting shifts in gut microbiota composition (Borre et al., 2014; Cowan et al., 2019) and exposure to ABX during adulthood drastically shifts gut microbiota composition and structure (Cox et al., 2014; Hoban et al., 2016; Leclercq et al., 2017; Ruiz et al., 2017). It is not surprise that the rearrangement of the microbiota composition is age dependent (Boehme et al., 2019). Increased stability and maturity of the gut microbiota in adulthood is likely to have prevented long-lasting effects of ABX_{adulthood} treatment and buffers some effects of ABX treatment, which could explain why behavioural and gene expression changes were only observed in ABX_{adolescence} mice.

Many studies have described the interaction between gut microbiota depletion either by ABX treatment or in GF mice and alterations in behaviour (Desbonnet et al., 2015; Frohlich et al., 2016; Hoban et al., 2016; Cryan et al., 2019). Here, we observed a statistically significant increase in anxiety-like behaviour in the EPM in ABX_{adolescence} mice but not in ABX_{adulthood} mice, reproducing the pattern that was observed for changes in microbiota composition and structure. The role of gut microbiota depletion in anxiety, however, is controversial with studies describing a reduction of anxiety-like behaviours (Bercik et al., 2011; Desbonnet et al., 2015), no effect (Frohlich et al., 2016; Hoban et al., 2016) or an anxiolytic effect following microbiota depletion (Leclercq et al., 2017). Firmicutes, Actinobacteria and Bacteroides are known to affect anxiety levels, with lower abundance being associated with elevated anxiety-like behaviour in rodents and humans (Bercik et al., 2011; Bravo et al., 2011), which is in line with the changes in gut microbiota composition observed in ABX_{adolescence} mice. In particular, we found *Lactobacillus acteroideus* and *Lachnospiraceae* NKA136 reduced in ABX_{adolescence} mice.

Indeed, the stronger effect observed in adolescent mice over adults may have been influenced by sex hormones peaking during adolescence. Preclinical studies in males suggest that testosterone yields protective benefits against anxiety [79] while decreased levels of testosterone is associated with significantly higher prevalence of anxiety disorders in men, compared to those with normal levels [80–82]. Moreover, high levels of testosterone during the adolescence is known to influence the commensal bacteria Firmicutes/Bacteroides ratio [8, 67, 68]. Therefore, the depletion of the gut microbiota during a critical developmental window itself has impaired the natural interaction between the testosterone and commensal bacteria that, together with the enduring shift in microbiota composition and structure are likely to have contributed to the increased anxiety-like behaviour in ABX_{adolescence} mice.

In addition, it has previously been found that administration of probiotics containing *Lactobacillus* (Firmicutes) correlates with the expression of *Gabrb1* within the amygdala, an effect that has been shown to be influenced by vagal communication [49, 83]. Given that *Gabrb1* is strongly reduced in mice treated with ABX during adolescence but not during adulthood this data suggest that alterations in gut microbiota composition induced by ABX treatment plays a role in GABA receptor expression in the amygdala which translates in behavioural alterations.

Interestingly, despite the body of literature showing that the gut microbiota is implicated in social behaviour or cognitive abilities [28, 36–38, 60, 64, 84–88], we did not observe any overt effect of ABX treatment in the three chamber task nor the novel object recognition task suggesting that with the parameters we used these phenotypes are not responsive to long-lasting effects of gut microbiota depletion. Moreover, it has been shown that deficient synaptic pruning, which is associated with weak synaptic transmission and decreased functional brain connectivity is correlated with deficits in social interaction and other neuropsychiatric disorders [89]. These failure in the synaptic connectivity is due to lack of activity of the *Cx3cr1* gene [89, 90]. Since *Cx3cr1* here was found upregulated, this is probably play locally role on brain inflammatory response which is not directly associated with sociability. In addition, fear conditioning which has previously been shown to be modulated by microbiome manipulations [42, 49, 91] was only marginally affected by gut microbiota depletion as only ABX_{adolescence} mice showed increased freezing behaviour during fear acquisition, indicating that learning and memory indeed is not overtly influenced by ABX treatment.

Changes in gut microbiota composition and structure have been linked to changes in gene expression in the brain [92]. Here we focused on the amygdala to understand to which extent changes in gene expression could underly the differences observed in the anxiety-like behaviour. Gene expression in the amygdala was more drastically affected in ABX_{adolescence} mice, with twice as many genes of those tested affected compared to ABX_{adulthood} mice. Despite the highest prevalence of overall gene expression changes in ABX_{adolescence}, the administration of ABX induced similar disturbance for genes involved in neuroimmunity reponse in ABX_{adolescence} and

ABX_{adulthood} mice. Gene expression of various genes related to microglial-mediated phagocytosis and components of the innate immune system were strongly affected. Given the importance of microglia in the normal brain function, it is not surprising that these genes have been associated with the ABX treatment. The microglia-related genes *Fcgr2b* and *Rac2* were upregulated in both groups, while *Cx3cr1* and *C1qa* were elevated only in ABX_{adolescence} mice, confirming a role for microbiota recolonization observed in ABX_{adulthood} (but not in ABX_{adolescence}) with restored microglia features [90]. It has been suggested that elevated levels of the *Cx3cr1* ligand CX3CL1 is associated with pro-neurogenic response by altering the environment in which new cells are born [93], suggesting that together with *C1qa*, *Cx3cr1* are playing a role in the prevention of chronic inflammation induced by the gut microbiota depletion. In fact, microglia seems to keep engulfing apoptotic debris even after antibiotic treatment [94], which together with elevated *Tlr4* expression followed by ABX_{adolescence} treatment could indicate certain level of neuroinflammation has been occurring without the participation of the cytokines. In addition, it is well known that SCFAs directly affect immune response with the intention to maintain homeostasis by finetuning microglial function and production of inflammatory cytokines, further suggesting that the overexpression of the SCFAs transporters *Slc5a8* and *Slc16a1* in the amygdala of ABX_{adolescence} mice could be related with the microbiome-derived factors that are involved in modulate adaptive immune responses in the brain.

C3 is a downstream member of the component cascade with an extremely versatile role that it is not restricted to immune responses but also in tissue regeneration and synapse pruning [95]. Interestingly, *C3* and *Il1b* were elevated only in ABX_{adulthood}. This could suggest that neuronal loss and consequently cognitive deficits associated with long-term depression caused by *C3* could have been counteracted by production of *Il1b*, a cytokine with a neuromodulatory role on neurogenesis [96].

Tight junction proteins at the blood-brain barrier are known to be regulated by the gut microbiota, where the decreased expression of these proteins are associated with an increase in blood-brain barrier [97–99]. Although gut depletion is necessary to promote tight junction protein changes in the brain, the direction of these changes is dependent of the brain region and protein tested. Herein, we observed an

upregulation of amygdalar *Tjp1* and *Ocln* only in ABX_{adolescence} mice, confirming the role of depleted gut microbiota on brain permeability [37, 99]. Moreover, this study also showed that natural recolonization of the gut microbiota is enough to normalize the functionality of the information transfer between the gut and brain. Taken together, our findings show that the immunological markers changed in ABX_{adolescence} and ABX_{adulthood} mice are not a result of a brain leakage through the blood-brain barrier but probably due to local synthesis or volume diffusion into the brain at the circumventricular organs that lack a blood-brain barrier [100, 101].

When examining the pattern of expression of genes involved in neurotransmission it was clear that they were more strongly affected in ABX_{adolescence} than ABX_{adulthood} mice. *Gabra2*, *Gabrb1*, *Grm5*, *Npy*, *Npy1r* and *Nr3c1* were upregulated in the amygdala of ABX_{adolescence} mice, while *Crh1r* was the only gene affected in ABX_{adulthood} mice. Elevated levels of *Crh1r* in brain regions like the hypothalamus and the hippocampus are usually found immediately after ABX treatment or in GF mice [12, 38] while downregulation of the transcription levels of *Crh1r* has been found after biotherapeutics or SCFA treatment in stressed animals [102, 103] and could explain why we see altered *Crh1r* expression in ABX_{adulthood} mice. In addition, NPY and activation of NPY1R are known to promote stress resilience and have anxiogenic effects [104–109]. NPY locally influences GABAergic activity through NPY1R, and both were found to be upregulated in chronic stress events [108, 109], in line with the results observed in ABX_{adolescence} mice. In fact, evidence suggest that the gut microbiota may contribute to resilience after repeated stress [110–113]. For instance, it has been shown that chronic stress significantly improve the gut microbiota diversity in antibiotic-treated mice and neutralize not only stress-inducing anhedonia phenotype as well blood levels of inflammatory markers, suggesting that the development of susceptibility to stress in mice is subjected to the gut microbiome composition [110, 113].

Interestingly, genes related with neuroplasticity, postsynaptic density protein 95 (*Psd95*) and synaptophysin (*Syp*) were oppositely affected by ABX_{adolescence} and ABX_{adulthood}, suggesting a direct role of the gut microbiota composition on the expression of these genes. A recent study has shown that depletion of the gut

microbiota drastically elevated the levels of such genes in the brain [113], whereas restoration of the gut microbiota normalizes the levels of *Syp* and *Psd95* [15]. Therefore, the upregulated expression of these genes in ABX_{adolescence} mice could be due to the long-lasting shift in microbiota composition and structure, whereas the decrease observed in ABX_{adulthood} mice might come from an exaggerated downregulation of these genes due to a possibly overexpression during the gut microbiota depletion period.

Unexpectedly, ABX_{adulthood} treatment seems also to play a role in myelin-related gene. Recent studies have shown upregulation of myelination-related genes in the PFC of animals with disrupted gut microbiota, suggesting a role for the gut microbiota in the formation of myelin [50, 114]. Our study indicates that depletion of the gut microbiota does not exclusively affect myelination during critical developmental periods but can also influence myelination-related gene expression in adulthood. Whether these changes in gene expression translates to altered myelination, however, still needs to be investigated.

Overall, this study highlights vulnerability of the gut microbiota during the adolescent period and the importance of the microbiota during the developmental period in shaping its host's microbiota, brain and behaviour. Adolescence is a time where the body and brain are in dramatic shift, facing a handful of stressful experiences such as unstable hormones and changes in functional connectivity in the brain. Taking into account the role of the gut microbiota in the brain and sexual hormones, adolescence is a sensitive period where minimal interventions have a huge impact that may be carried to life. Future studies are needed to determine if the behavioural effects are driven by the discrete genes in gene expression in the amygdala. Further, understanding what pathways of communication between the gut and the brain are responsible for such changes at this time period are also required (eg. vagus nerve) [115, 116]. Moreover, whether the effects seen can be reversed, ameliorated or even worsened by specific dietary interventions would be of further interest to investigate [40, 117]. Finally, as adolescence is a time for antibiotic usage clinically [118] and its use is epidemiologically relevant to the risk of several psychiatric conditions [119], the results presented here support the need for further studies investigating their

impact and their long-term effects on the microbiota and associated risks to the brain function and behaviour.

3.3 Strategies for effective gut microbiota recovery after chronic broad-spectrum antibiotic administration in adult male rats.

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3.3.1 Abstract

There is considerable interest in the contribution of microbiota-gut-brain signalling in integrative physiological systems, which has recently extended to cardiorespiratory control. Chronic antibiotic strategies to reduce the microbiota are commonly employed. However, the optimal approach to normalise the gut microbiota, and interactions that occur during efforts to recolonise the gut microbiota after significant microbial perturbations, are currently unknown.

In adult male rats, we sought to characterise the effects of prebiotics, faecal microbiota transfer and natural recolonisation on the gut microbiota after 4-week administration of a cocktail of antibiotics or vehicle. 16S sequencing of the caecal contents was performed for microbiota analysis. Furthermore, high-performance liquid chromatography and whole-body plethysmography was used to assess brainstem neurochemistry and respiratory control, respectively.

Using a compositionally appropriate approach, an interaction was found between antibiotics and prebiotics for the colonisation rate of *Blautia*, as well as five functional modules in the microbiota. *Blautia* was found to be responsible for a large partition of these modules. Modulation of the gut microbiota caused significant disruptions to brainstem monoamine neurochemistry, but had no major effect on respiration during room air breathing or in response to hypoxic or hypercapnic chemostimulation.

Natural recolonisation appears the optimal approach to restore the gut microbiota following antibiotic administration in rodents. *Blautia* was shown to only colonise the caecum following antibiotic administration, in the subsequent presence of prebiotics. We posit that these findings have significant implications for studies employing manipulation of the gut microbiota, with potential relevance to clinical settings.

3.3.2 Introduction

The gut microbiota defines an extensive community of numerous commensal, pathogenic and symbiotic microbes that reside within the gastrointestinal tract. This ecosystem includes bacteria, archaea, viruses and fungi. It is estimated that the gastrointestinal bacterial population establishes a ratio of host:bacterial cells 1:1 (Sender et al., 2016). The gut microbiota is influenced throughout life by various factors including life-style, hormones, ageing, stress, health status, environment, medication, amongst others (Woodmansey, 2007; O'Mahony et al., 2011; Albenberg and Wu, 2014; Panda et al., 2014; Golubeva et al., 2015; Kelly et al., 2015; Foster et al., 2017; Boehme et al., 2019). Extensive evidence suggests that the gut microbiota plays a fundamental role in whole-body health and disease (Cryan and O'Mahony, 2011; Grenham et al., 2011; Kelly et al., 2016; Sarkar et al., 2016; Kelly et al., 2017). Studies in rodents display that aberrant gut microbiota is associated with numerous maladies, including anxiety, depression and cardiorespiratory pathologies (Kelly et al., 2015; Burokas et al., 2017; Ganesh et al., 2018; O'Connor et al., 2020). Similarly, disrupted gut microbiota is increasingly associated with a broad range of human diseases such as cardiovascular disease, obesity and inflammatory bowel disease/syndrome (Benjamin et al., 2012; Burke et al., 2017; Yan et al., 2017). It is widely accepted that antibiotic administration, in rodents and humans, damages the natural gut microbiota composition and diversity (Blaser, 2016; Gasparrini et al., 2016; Haak et al., 2019; O'Connor et al., 2019). Despite this, best practice for remediation of the antibiotic-depleted gut microbiota in rodent models and in standard clinical practice is unknown (Gagliardi et al., 2018; O'Connor et al., 2019; Wilson et al., 2019).

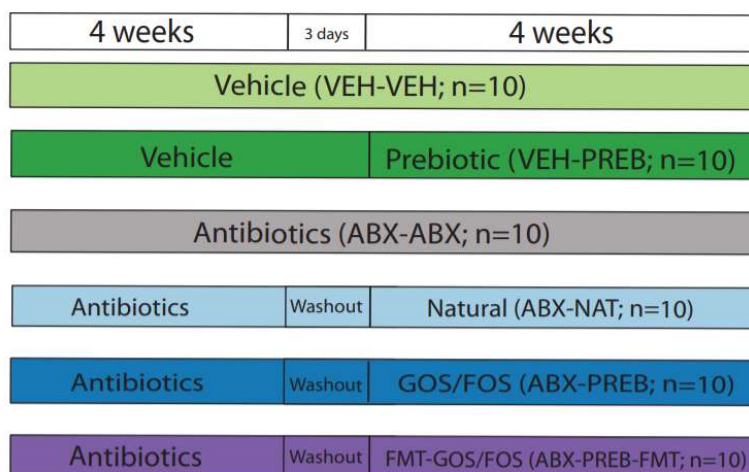
In rodents, broad spectrum antibiotics are routinely used to investigate the role of a depleted gut microbiota on integrative body systems (Hoban et al., 2016; Galla et al., 2018; O'Connor et al., 2019). Normalisation or beneficial expansion of the gut

microbiota following antibiotic administration would benefit investigations aiming to tease out associations between the gut microbiota and brain/physiological functions. Furthermore, mounting evidence suggesting a casual role of aberrant gut microbiota in disease has led to the necessity to develop targeted therapeutic approaches to restore or beneficially promote the microbial composition. Among these, faecal microbiota transfer (FMT) has demonstrated a profound capability to shift the gut microbiota in rodents and in patients (Mullish et al., 2015; Suez et al., 2018; Wilson et al., 2019). FMT studies in rodents, using a variety of transplantation methods, aim to assess if 'restoration' of the gut microbiota reverses abnormal brain behaviours, such as depression, anxiety and cardiorespiratory impairments (Kelly et al., 2015; Burokas et al., 2017; O'Connor et al., 2019). In recent years, FMT has been widely used as a treatment for recurrent *Clostridium difficile* infections in patients, and advances have been made in other chronic diseases (Wilson et al., 2019). Prebiotics are another method of microbiota modulation. Prebiotic fibres are fermented in the gut and produce specific changes in bacterial composition, promoting the growth of beneficial gut microbes (Gibson et al., 2010). Prebiotics have recently been included in the definition of 'psychobiotics' and have effects on emotional, central and systemic functions in rodents and in clinical populations (Sarkar et al., 2016). We explored whether the gut microbiota, depleted as a result of broad spectrum antibiotics, could be restored or beneficially expanded through the use of intervention strategies known to modulate the gut microbiota and/or by natural recolonisation. Furthermore, we examined the effects of altered gut microbiota on the control of breathing, given that we previously reported that disruption to the gut microbiota was associated with respiratory dysfunction, which was related to reported disturbances in brainstem neurochemistry (O'Connor et al., 2019).

3.3.3 Materials and Methods

3.3.3.1 Ethical approval

Procedures on live animals were conducted under licence from the Government of Ireland Department of Health (B100/4498) in accordance with National and European directive 2010/63/EU. Ethical approval was obtained from University College Cork (AEEC #2013/035) and procedures were carried out in accordance with guidelines laid down by University College Cork's Animal Welfare Body.



Antibiotic cocktail: Ciprofloxacin, imipenem, ampicillin, vancomycin and metronidazole

FMT: 2 x oral gavage of pooled faeces from 3 VEH rats

ABX-PREB and ABX-PREB-FMT cages received bedding from VEH-PREB and VEH cages during recolonisation

Fig. 3.3-1 Experimental setup. Schematic representation of the experimental design utilised in this study.

3.3.3.2 Experiment animals

Ten-week old adult male Sprague Dawley rats (n=60; purchased from Envigo, UK) were housed as age-matched pairs in standard rat cages under a 12-hour light: 12-hour dark cycle. Rodents had *ad libitum* access to standard rat chow.

3.3.3.3 Antibiotic administration

Rats were randomly allocated to receive autoclaved deionised water (vehicle, VEH; n=30) or a cocktail of antibiotics (ABX; n=30) for 4-weeks. In an effort to deplete the microbiota, broad-spectrum antibiotics including ciprofloxacin (20 mg/l), imipenem (250 mg/l), ampicillin (1 g/l), vancomycin (500 mg/l) and metronidazole (1g/l) in autoclaved deionised water were utilised. Water bottles were replenished every second day. Rat body weights, water consumption and food intake were recorded every other day. Fresh bedding was transferred to ABX rats every second day.

3.3.3.4 Collection and processing of microbiota for faecal microbiota transplantation

Faecal microbiota was obtained from the pooled faeces of 3 VEH rats. Faeces was collected and processed as previously described (O'Connor et al., 2019).

3.3.3.5 Prebiotic and FMT administration

Rats consuming broad spectrum antibiotics for 4-weeks remained on antibiotics for an additional 31 days (ABX-ABX; n=10) or received a washout period of autoclaved deionised water for 3 days. Following the washout period, rats remained on autoclaved deionised water (ABX-NAT; n=10) or were transferred to prebiotics (PREB; 7.5 g/L of galactooligosaccharides and fructooligosaccharides) in autoclaved deionised water for 4-weeks, with or without FMT administration (ABX-PREB-FMT, n=10; ABX-PREB, n=10, respectively), as previously described (O'Connor et al., 2019; O'Connor KM, 2020). After 31 days, rats assigned to vehicle administration continued to receive water (VEH-VEH; n=10) or were transferred to prebiotics (VEH-PREB; n=10) for 4-weeks. Rat body weights, water consumption and food intake were recorded every other day. Fresh bedding was transferred to ABX-ABX cages every second day. Bedding was changed for all other cages weekly; VEH-VEH, VEH-PREB and ABX-NAT cages were transferred to fresh bedding. In order to promote host microbiota establishment, ABX-PREB and ABX-PREB-FMT cages received bedding from VEH-PREB and VEH cages, respectively.

3.3.3.6 Assessment of respiratory flow and metabolism in the unrestrained rat during quiet rest

3.3.3.6.1 Whole-body plethysmography

In unrestrained rats during quiet rest, whole-body plethysmography (DSI, St. Paul, Minnesota, USA) was used to record respiratory flow recordings. Rats were introduced into custom plethysmograph chambers (601-1427-001 PN, DSI). Room air moved through the chambers (3l/min) ensuring adequate oxygen (O_2) and carbon dioxide (CO_2) environmental conditions. Rats were allowed to settle for 30-90 min to encourage habituation to the surroundings.

3.3.3.6.2 Metabolic measurements

CO_2 production (VCO_2) and O_2 consumption (VO_2) were measured in rats throughout the protocol (O_2 and CO_2 analyser; AD Instruments, Colorado Springs, CO, USA) as previously described (Haouzi et al., 2009; Bavis et al., 2014; Lucking et al., 2018).

3.3.3.6.3 Experimental protocol

Following the acclimation period, baseline parameters were assessed during a 10-15-min steady-state normoxia period ($FiO_2 = 0.21$; balance N_2). This was followed by a 10-min poikilocapnic hypoxia challenge ($FiO_2=0.10$; balance N_2). Each rat was re-exposed to normoxia to re-establish stable basal breathing. Subsequently, animals were exposed to a 10-min hypercapnia challenge ($FiCO_2 = 0.05$; balance O_2) followed by a recovery period.

3.3.3.6.4 Data analysis for whole-body plethysmography

Respiratory variables including respiratory frequency (f_R), minute ventilation (V_I), tidal volume (V_T), expiratory time (T_e) and inspiratory time (T_i) were recorded on a breath-by-breath basis for analysis (FinePointe software Buxco Research Systems, Wilmington, NC, USA). Artefacts in respiratory flow signals relating to animal movement and sniffing were omitted from analysis. A steady-state normoxia period

was averaged to assess baseline respiratory and metabolic parameters. Ventilatory and metabolic data were reported for the final 5-mins of the acute poikilocapnic hypoxic and hypercapnic challenges allowing for adequate gas mixing in the plethysmograph chambers ensuring steady-state assessment of respiratory and metabolic parameters. Data are expressed as a change in absolute from baseline values. Respiratory flow signals were examined for the occurrence of augmented breaths (sighs) during normoxia, hypoxia and hypercapnia as well as for apnoea events (spontaneous and post-sigh apnoeas) during normoxia as previously described (Edge et al., 2012). The criterion for apnoea was a pause in breathing for a duration greater than two consecutive missed breaths. Apnoea data are expressed as apnoea index (apnoea events per hour). A sigh was defined as an augmented breath twice the amplitude of the average V_T . Poincaré plots expressing breath-to-breath (BB_n) versus subsequent breath-to-breath interval (BB_{n+1}) were constructed allowing for determination of respiratory timing variability during steady-state baseline bouts and hypoxic and hypercapnic challenges. V_T , V_i , V_T/T_i , VO_2 and VCO_2 were normalised per 100g body mass.

3.3.3.7 Tissue collection

Animals were euthanised by decapitation under isoflurane anaesthesia (5% by inhalation in room air). Blood was collected immediately, prepared in 3% Na_2EDTA (disodium salt dehydrate) and centrifuged (14,000g for 20 min at 4 °C) for subsequent analysis. Plasma was snap frozen in liquid nitrogen. Whole brains were harvested and frozen at -80°C isopentane for subsequent high-performance liquid chromatography analysis. The lungs were removed and weighed and were allowed to air dry at 37°C for at least 48 h and re-weighed. The faeces from the colon as well as the caecum were harvested. The caecum was weighed and caecal contents were quickly removed and snap frozen in liquid nitrogen. The heart was removed and the right ventricle and left ventricle and septum were separated and weighed.

3.3.3.8 Brainstem monoamine concentrations

3.3.3.8.1 High-performance liquid chromatography (HPLC) coupled to electrochemical detection for measurement of pons and medulla oblongata monoamine concentrations

The brainstem was dissected from the brain and separated into two distinct regions, pons and medulla oblongata at -20°C. The pons and medulla oblongata were sonicated (Bandelin Sonolus HD 2070) in 1 ml of chilled mobile phase, spiked with 2ng/20µl of a N-methyl 5-HT (internal standard). High-performance liquid chromatography coupled to electrochemical detection was performed as previously described (Lucking et al., 2018; O'Connor et al., 2019). Noradrenaline (NA), dopamine (DA), serotonin (5-HT), monoamine precursor L-3,4 dihydroxyphenylalanine (L-DOPA) and metabolites 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were quantified. Each monoamine, precursor and metabolite was identified by its characteristic retention times. Standard injections were run at intervals during sample analysis.

3.3.3.8.2 Data analysis

Chromographs were processed using Class-VP5 software. Concentrations of monoamines, precursors and metabolites were determined using analyte:internal standard peak response ratios. Data are expressed as nanograms of neurotransmitter per gram of tissue weight (ng/g).

3.3.3.9 Plasma corticosterone concentrations

Plasma samples were thawed and concentrations of corticosterone were determined using commercially available enzyme-linked immunosorbent assay kit (ENZO Life Sciences, UK) according to the manufacturer's instructions. A spectrophotometer (SpectraMax M3, Molecular devices) was used to read absorbance for the assay.

3.3.3.10 16S rRNA sequence-based microbiota composition and diversity analysis in caecal content

3.3.3.10.1 DNA extraction from caecal matter

DNA was extracted from caecal matter as previously described (Gough et al., 2018).

3.3.3.10.2 16S rRNA Gene Sequence-based microbiota analysis.

Primers were used to prepare the 16S metagenomic libraries by amplifying the V3-V4 region of the 16S gene, with Illumina adaptors incorporated as described in the Illumina 16S Metagenomic Library preparation guide with the following exceptions: the first PCR reaction was performed in a total volume of 50µl instead of 25µl and the number of cycles used in the first PCR was increased from 25 cycles to 30. The volume of AMPure XP beads (NEB) used in the initial clean-up was scaled up accordingly. Following index PCR and purification, Qubit high sensitivity DNA kit (Life technologies) was used to quantify the products. The PCR products were pooled equimolarly. The pooled libraries were assessed by Agilent high sensitivity DNA kit and quantified by qPCR using the Kapa Quantification kit for Illumina (Kapa Biosystems) according to the manufacturer's guidelines. Libraries were diluted and denatured following Illumina guidelines and sequenced on the Illumina MiSeq using the V3 600 cycle kit according to Illumina sequencing protocols at the Teagasc sequencing facility, Teagasc Food Research Centre, Fermoy, County Cork, Ireland.

3.3.3.10.3 Bioinformatic sequence analysis

The resulting amplicons after sequencing with the MiSeq Illumina platform (2x300pb paired-end reads) were pre-filtered based on a quality score threshold of >28 and trimmed, filtered for quality and chimaeras using the DADA2 (Callahan et al., 2016) library in R (v3.6.3). Taxonomy was assigned with DADA2 against the SILVA SSURef database release v132. Parameters recommended in the DADA2 manual were adhered to. Amplicon Sequence Variants (ASVs) were aggregated at genus level, those that were unknown on the genus level were not considered in downstream

analysis, as were genera that were only detected as non-zero in five per cent or fewer of total samples.

3.3.3.10.4 Data analysis

Microbiome based data analysis was performed in R (v3.6.3) in the Rstudio environment (v1.2.5033). The ALDEx2 (Fernandes et al., 2014) library in R was used to perform a center log-ratio (clr) transformation in order to account for the compositional nature of microbiome data sets. Alpha diversities were calculated using the iNEXT (Hsieh et al., 2016) library. Beta diversity was calculated as Aitchison distance and differences were assessed by PERMANOVA followed by a pairwise implementation of PERMANOVA as a post-hoc. Functional inference was performed by use of the online Phiphillin service (Iwai et al., 2016). The resulting count table of Kegg orthologues was piped into the omixerRpm R library in order to detect gut-brain modules (GBMs) and gut-metabolic modules (GMMs)(Valles-Colomer et al., 2019). Custom R scripts and functions are publically available online (Bastiaanssen, 2018). In all cases, FDR was controlled by Benjamini Hochberg, with a threshold of 20%.

3.3.3.11 Universal 16S bacterial PCR in faecal matter

DNA was extracted from faecal material of ABX-ABX and VEH-VEH rats using QIAamp Fast DNA Stool mini kit (Qiagen), following the manufacturer's protocol. An additional bead-beating step was added to increase DNA yield. Qubit dsDNA broad range kit was used to quantify dsDNA. The final 16S PCR reaction mix (0.2µl F: 5'-CGGCAACGAGCGCAACCC-3' (1114) (10 mM work solution), 0.2µl R: 5'-CCATTGTAGCACGTGTGTAGCC-3' (1275) (10 mM work solution), 5µl KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal and DNA 10ng/well diluted in PCR H₂O) was 10µl/well. RT-PCR was carried out using a LightCycler 96 (Roche Diagnostics Ltd.) on a 96-well plate. All reactions were performed in triplicate. Relative number of copies were calculated using the 2^{-Ct} method. Data were expressed as fold-change ratio: ABX-ABX/VEH-VEH.

3.3.3.12 Statistical analysis

Data were assessed for outliers, normal distribution and equality of variances using box-plots, Shapiro-Wilk test and Levene's test, respectively. For data sets with confirmed normal distribution, a one-way ANOVA with Dunnett's test was used to test for statistically significant between group differences. When data were normally distributed, but the assumption of homogeneity of variances was violated, a Welch ANOVA with Games-Howell test was used. For data sets where normal distribution was violated, non-parametric Mann-Whitney *U* test or Kruskal-Wallis test with Mann-Whitney *U* test for pairwise comparisons was used to test for statistical significance. Statistical significance was assumed at $p < 0.05$. Bonferroni correction was applied to adjust for multiple comparisons, with the exception of microbiota data. The ALDEx2 R library was used for the generalised linear model (GLM) and differential abundance testing for microbiome features. Benjamini-Hochberg (BH) adjustment procedure was applied with the false discovery rate (FDR) set at 20% to correct for multiple testing in the clr-transformed microbiota data. Microbiota data are expressed as median (IQR). All other data are expressed as mean \pm SD or displayed graphically as box and whisker plots (median, IQR and minimum to maximum values). SPSS v25 was used for all other statistical analysis. GraphPad Software v6 (GraphPad Software, San Diego, CA, USA) and R software environment were used to generate graphs. Adobe illustrator CS5 (v15) and Inkscape were used to edit Figures.

3.3.4 Results

3.3.4.1 Body and tissue weight

Microbiota modulation had significant effects on body weight, right and left ventricle weights, and caecum and dry lung weights (Table 1). ABX-ABX and ABX-PREB rats had reduced body weight. Furthermore, heart weight was decreased in ABX-ABX rats. As expected, ABX-ABX had significantly heavier caecum, which was similar to previous studies (Hoban et al., 2016; O'Connor et al., 2019). Similarly, prebiotic administration significantly increased caecum weights in all groups due to heightened fibre intake. Natural recolonisation normalised caecum weight of rats previously treated with ABX.

3.3.4.2 Caecal microbiota composition and diversity

ABX-ABX rats had significantly depleted 16s bacterial DNA compared with VEH-VEH rats (data not shown) and perturbed gut microbiota (Supplementary excel). In terms of alpha diversity, beta diversity (Fig. 3.3-2a-b) and differentially abundant genera (Supplementary Fig. 1a, Fig 2c), naturally recolonised rodents (ABX-NAT) showed no differences compared to VEH (Fig 3.3-2a-c, Suppl Fig. 1a). Both ABX-PREB and VEH-PREB showed a similar change in terms of beta diversity (Fig. 3.3-2b). The ABX-PREB-FMT group showed the largest differences compared to VEH in alpha diversity, beta diversity and differential abundance (Fig. 3.3-2a-c and Supplementary Fig. 1a). After Bonferroni correction, ABX-PREB-FMT was lower than VEH in the Chao1 (Fig. 3.3-2a; $W = 7$, $p = 0.0049$) and Shannon (Fig. 3.3-2a; $W = 11$, $p = 0.021$) metrics. In terms of beta-diversity, all groups were different from VEH in a pairwise PERMANOVA (Fig. 3.3-2b; all $p < 0.001$, vs VEH-PREB $F = 3.35$, $R^2 = 0.157$; vs ABX-PREB $F = 4.15$, $R^2 = 0.19$; vs ABX-PREB-FMT $F = 12.11$, $R^2 = 0.40$) except for VEH vs ABX-NAT ($p > 0.05$; $F = 0.95$; $R^2 = 0.05$). In order to leverage the partial two-factor design of the study, we applied the ALDEx2 GLM implementation with the intent to test for interactions between ABX and PREB on a genus level. After the Benjamini-Hochberg procedure, only *Blautia* was found to have a significant interaction (Fig. 3.3-3a; $q = 0.072$, $t = -4.33$). Administration of PREB substantially increased the abundance of *Blautia* in the host, but the effect was much stronger following depletion of microbiota with ABX.

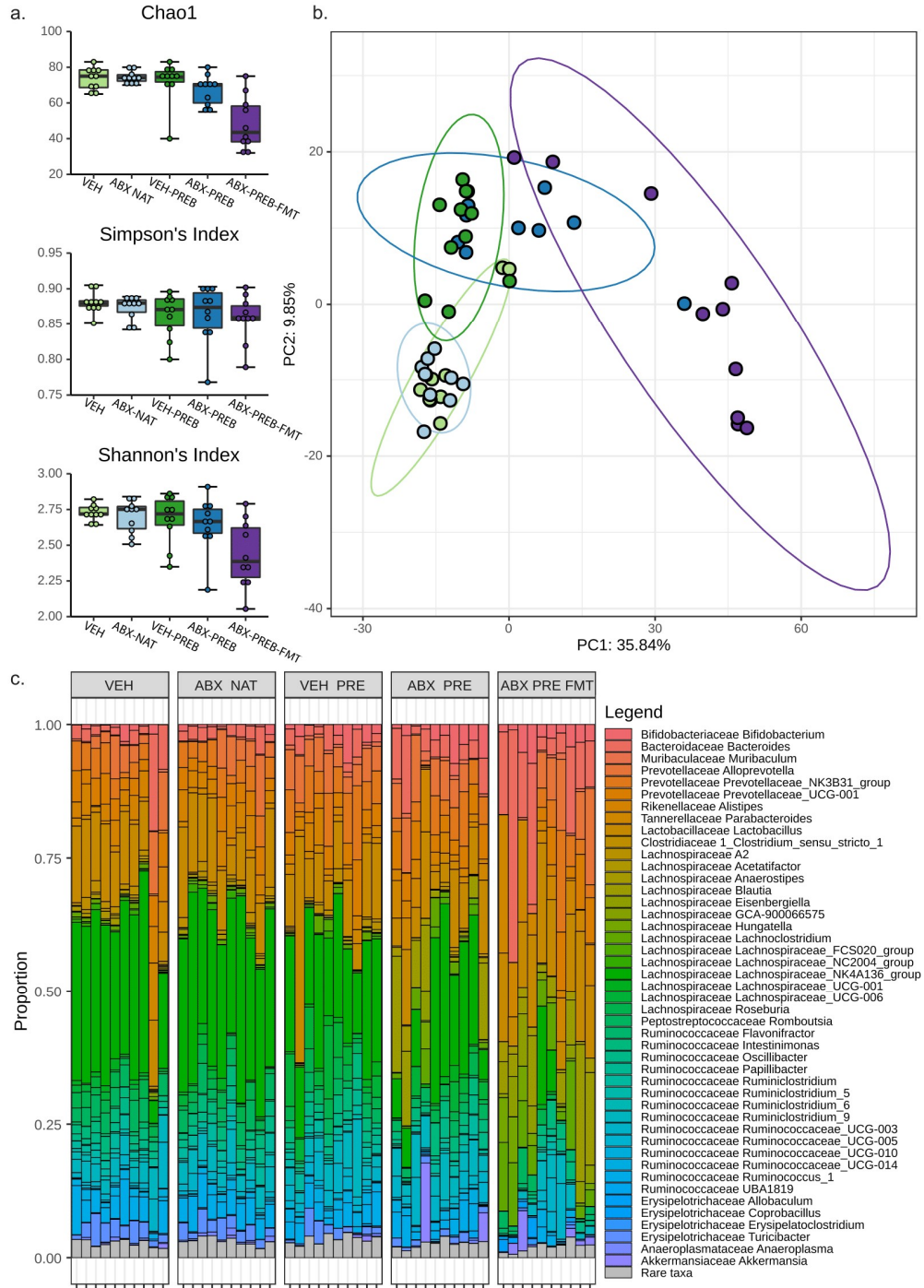


Fig. 3.3-2. Natural recolonisation normalised gut microbiota diversity. Group data for alpha diversity (a), principal component analysis (PCA; b) in 2-dimensional representation and stacked barplots showing taxa present at at least 0.1% at least once (c) for VEH-VEH, VEH-PREB, ABX-NAT, ABX-PREB and ABX-PREB-FMT. VEH, autoclaved deionised water; PREB, Prebiotic treated; ABX, antibiotic-treated; NAT, Natural recolonisation; FMT, faecal microbiota transfer. Data (a) are expressed as

box and whisker plots (median, IQR and minimum to maximum values). Data (a) were statistically compared by non-parametric Mann-Whitney U test.

3.3.4.3 Gut-brain modules and gut-metabolic modules

Pursuing the functional consequences of the difference in *Blautia*, we proceeded to detect affected gut-brain modules (GBMs) and gut metabolic modules (GMMs) with a similar pipeline to previous studies (Supplementary Fig. 1b, c) (Butler et al., 2020; Donoso et al., 2020). Using the same statistical procedure on the floored count tables, a significant interaction between PREB and ABX administration was found for two GBMs and three GMMs after post-hoc correction. Specifically, Inositol degradation; $q = 0.0747$; $t = -4.07$ and Glutamate degradation II; $q = 0.0288$; $t = -4.58$, for the GBMs (Fig. 3.3-3b, c) along with Glutamate degradation III; $q = 0.0468$; $t = -4.52$, Aspartate degradation II; $q = 0.1249$; $t = -3.47$ and Pyruvate:formate lyase; $q = 0.1031$; $t = -3.55$, for GMMs (Fig. 3.3-3b-f). These functional modules were increased by PREB, but the effect was substantially stronger following microbiota depletion by ABX. By tracing back the genomes in the KEGG database that contributed to these functions and aggregating these genomes on a genus level, we found that *Blautia* genomes contribute a substantial proportion of the detected 16S sequences that were inferred to be contributing to the GBMs and GMMs (Fig. 3.3-4a-e).

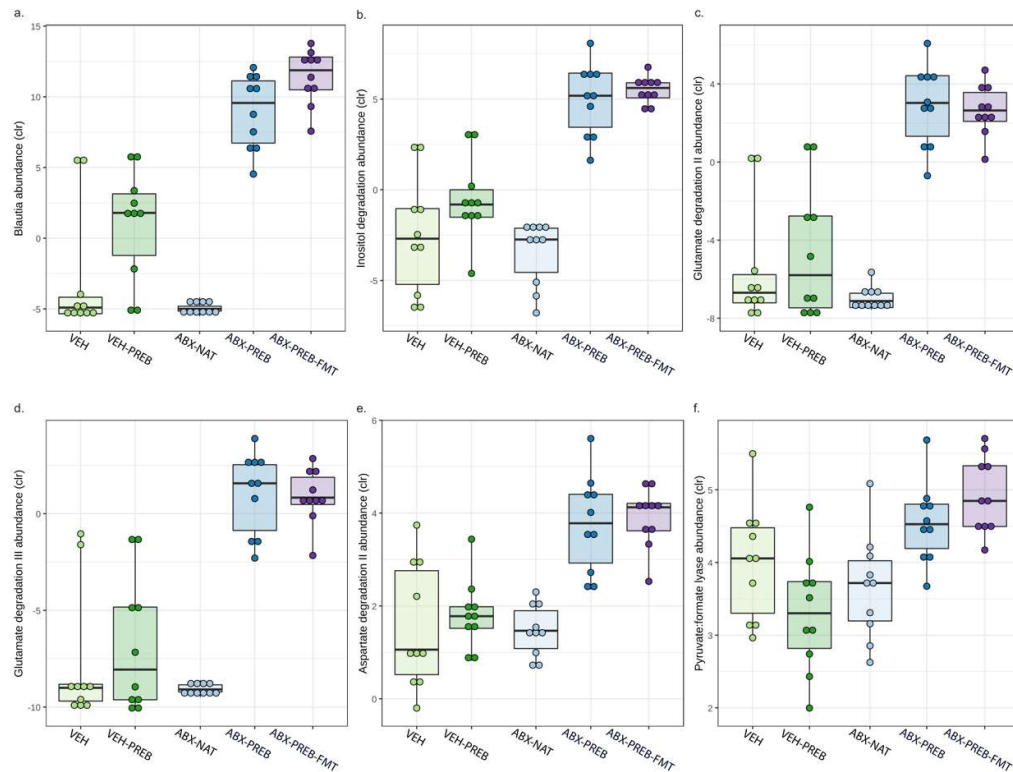


Fig. 3.3-3. Antibiotics and prebiotics interaction increases *Blautia* relative abundance and alters predicted function. Group data for *Blautia* relative abundance (a), inositol degradation abundance (b), glutamate degradation II abundance (c), glutamate degradation III abundance (d), aspartate degradation II abundance (e) and pyruvate:formate lyase abundance (f) in VEH-VEH, VEH-PREB, ABX-NAT, ABX-PREB and ABX-PREB-FMT. VEH, autoclaved deionised water; PREB, Prebiotic treated; ABX, antibiotic-treated; NAT, Natural recolonisation; FMT, faecal microbiota transfer.

3.3.4.4 Inflammatory markers and corticosterone

No statistically significant differences were evident in pro/anti-inflammatory cytokines and corticosterone plasma concentrations compared with VEH-VEH rats (Table 2).

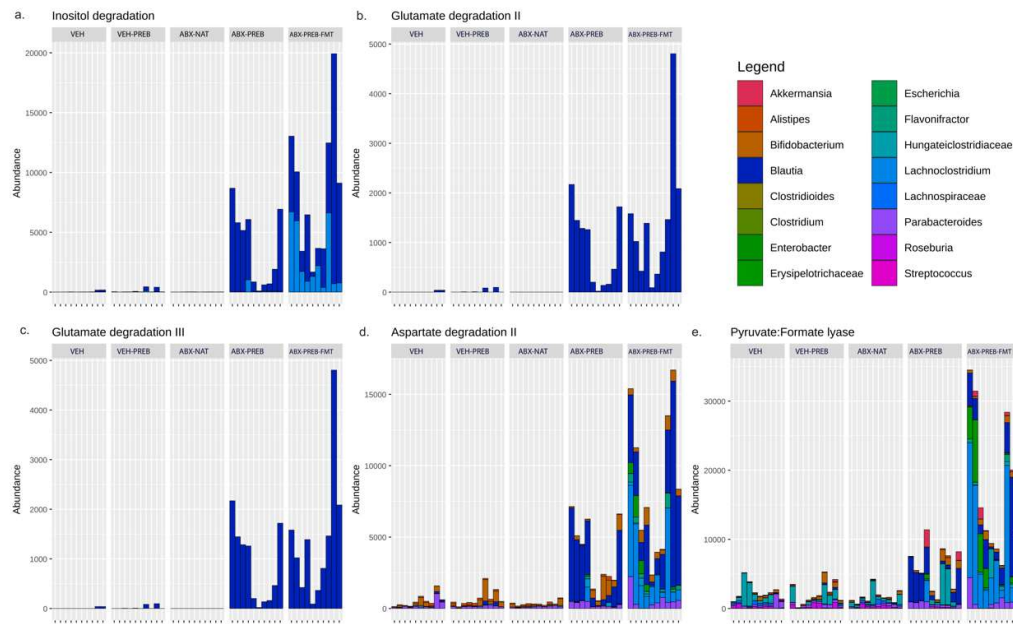


Fig. 3.3-4. *Blautia* relative abundance represents degradation and lyase of predicted functional characteristics in antibiotic-treated rats administered prebiotics. Bar columns for relative abundance of bacterial genera related to inositol degradation abundance (a), Glutamate degradation II abundance (b), Glutamate degradation III abundance (c), Aspartate degradation II abundance (d) and Pyruvate:formate lyase abundance (e) in VEH-VEH, VEH-PREB, ABX-NAT, ABX-PREB and ABX-PREB-FMT. VEH, autoclaved deionised water; PREB, Prebiotic treated; ABX, antibiotic-treated; NAT, Natural recolonisation; FMT, faecal microbiota transfer.

3.3.4.5 Monoamine concentrations in the pons and medulla oblongata

Comparisons of L-DOPA, HVA, HVA/DA, 5-HT, 5-HIAA and 5-HIAA/5-HT in the pons and medulla oblongata revealed significant differences between groups (Fig. 3.3-5a, c, d, f-h). Additionally, DA and NA were altered in the pons and medulla oblongata, respectively (Fig. 3.3-5b, e). *Post hoc* analyses revealed significant differences compared with VEH-VEH rats. Medulla oblongata NA concentrations were decreased in VEH-PREB rats ($p=0.004$). HVA concentrations in the pons ($p=0.002$) and medulla oblongata ($p<0.0001$) of VEH-PREB rats was significantly reduced, which resulted in blunted dopamine turnover (HVA/DA) in the pons ($p<0.0005$). ABX-ABX rats had diminished HVA ($p=0.004$) and HVA/DA ($p=0.002$) concentrations in pons and medulla oblongata regions. Elevated serotonin turnover (5-HIAA/5-HT) was evident in the medulla oblongata of ABX-ABX rats ($p=0.006$). Pontine HVA ($p=0.002$) and

medulla oblongata 5-HIAA ($p=0.001$) concentrations were decreased and increased, respectively in ABX-Nat rats. Pontine L-DOPA ($p=0.008$), HVA ($p=0.01$) and HVA/DA ($p=0.006$) concentrations were reduced in ABX-PREB; pontine DA ($p=0.001$) and medulla oblongata 5-HIAA/5-HT ($p=0.002$) concentrations were increased. ABX-PREB-FMT had elevated DA ($p=0.007$) and 5-HIAA ($p=0.007$) concentrations in the pons and medulla oblongata, respectively. Decreased dopamine turnover ($p=0.001$) and NA concentrations ($p=0.008$) were evident in the pons and medulla oblongata, respectively of ABX-PREB-FMT rats.

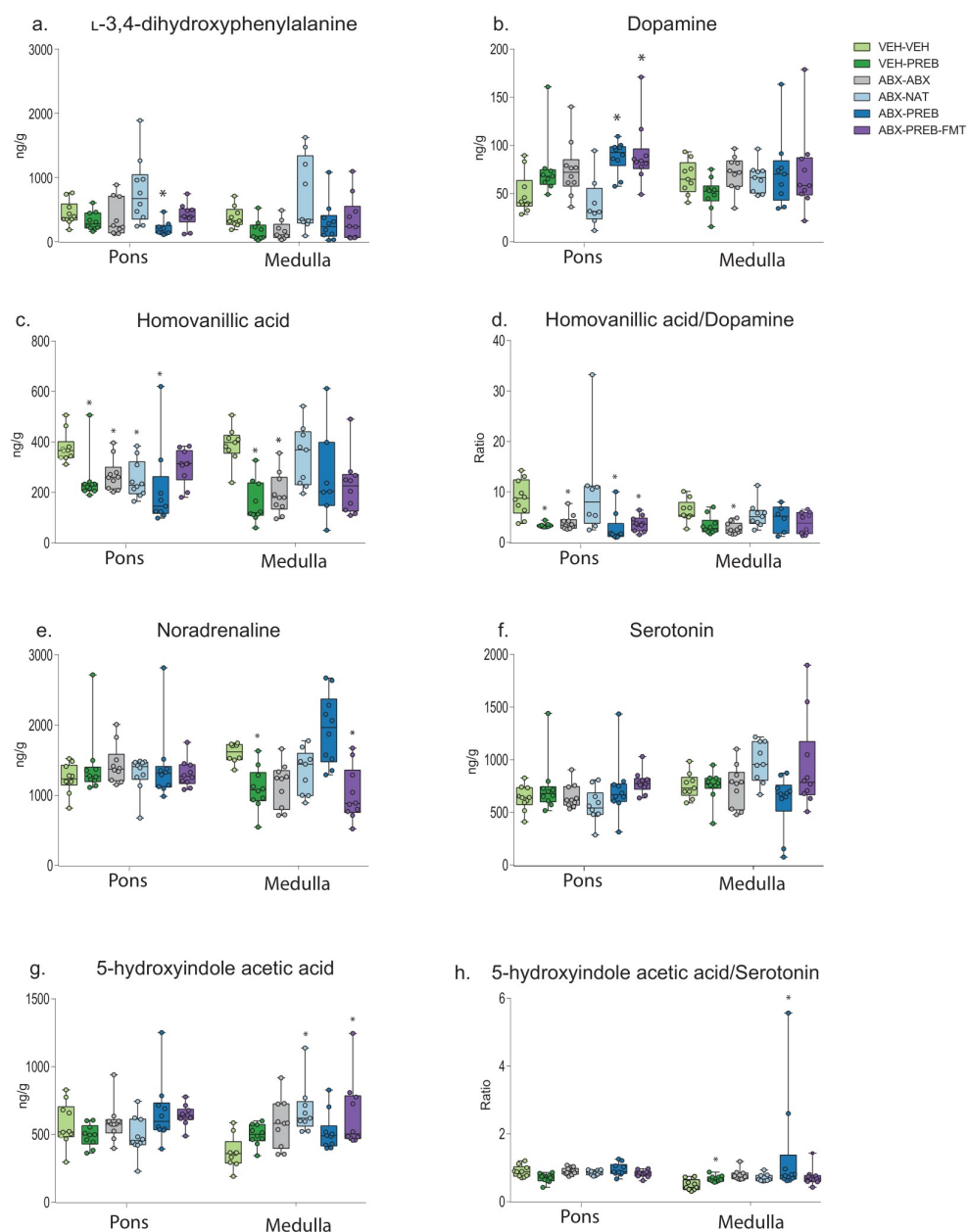


Fig. 3.3-5. Microbiota modulation alters pontine and medulla oblongata neurochemistry. Group data for L-3,4-dihydroxyphenylalanine (a), dopamine (b), homovanillic acid (c), homovanillic acid/dopamine ratio (d), noradrenaline (e), serotonin (f), 5-hydroxyindole acetic acid (g) and 5-hydroxyindole acetic acid/serotonin ratio (h) for pons and medulla in VEH-VEH, VEH-PREB, ABX-ABX, ABX-NAT, ABX-PREB and ABX-PREB-FMT rats. VEH, autoclaved deionised water; PREB, Prebiotic treated; ABX, antibiotic-treated; NAT, Natural recolonisation; FMT, faecal microbiota transfer. Groups (a-h) are expressed as box and whisker plots (median, IQR and minimum to maximum values). $n=7-10$. Groups were statistically compared using one-way ANOVA, Welch's ANOVA or non-

*parametric Kruskal-Wallis test. Dunnetts test, Games-Howell and Mann-Whitney U test, were used appropriate for post hoc analysis. * $p < 0.05$ divided by the number of comparisons made.*

3.3.4.6 Baseline, hypoxic and hypercapnic ventilation

During room air and hypoxic breathing, long-term breathing instability (SD2) was altered by microbiota manipulation (Supplementary Table 1). Drive to breathe (V_T/T_i) and inspiratory time (T_i) in response to hypercapnic chemostimulation were affected by microbiota manipulation; sigh amplitude during hypercapnia was also different between groups (Supplementary Table 1). Other respiratory parameters assessed during room air and in response to hypoxic and hypercapnic chemostimulation were unaltered (Supplementary Table 1, Supplementary Fig. 2 and 3; $p > 0.05$).

3.3.5 Discussion

A collective body of consensus-based evidence emphasises the regulatory role of the gut microbiota on whole-body function in health and disease states (Cryan and O'Mahony, 2011; Grenham et al., 2011; Kelly et al., 2016; Sarkar et al., 2016; Kelly et al., 2017). In rodent microbiota intervention studies (i.e. antibiotic administration and FMT), aberrant gut microbiota is a potent contributor to numerous maladies including depression, anxiety and cardiovascular pathologies (Kelly et al., 2015; Burokas et al., 2017; Ganesh et al., 2018). More recently, we have extended this line of enquiry by linking disturbed gut microbiota to altered control of breathing (Lucking et al., 2018; O'Connor et al., 2019). In humans, disrupted gut microbiota composition and diversity has been associated with multiple illnesses such as cardiovascular disease as well as neurodegenerative, neurodevelopmental, metabolic and biopsychosocial disorders (Benjamin et al., 2012; Burke et al., 2017; Yan et al., 2017). Given the rapid advancements in research regarding the potential of the gut microbiota to shape physiological and brain function there is increased necessity for the development of targeted approaches to restore or beneficially promote the gut microbiota.

We sought to explore whether disruption of the gut microbiota composition and diversity as a result of broad spectrum antibiotic cocktail administration could be normalised or beneficially expanded through natural recolonisation and/or the use of interventions known to alter the gut microbiota. Furthermore, we expand on present knowledge investigating the influence of the gut microbiota on the respiratory control system. The principal findings of this study are: 1) Following antibiotic administration, natural recolonisation led to a similar gut microbiota composition, diversity and predicted microbial function to that of vehicle treated animals; 2) Prebiotic administration modulated composition and function of the gut microbiota; 3) Prebiotic administration significantly expanded *Blautia* relative abundance in animals pre-treated with antibiotics; *Blautia* relative abundance was

associated with predicted gut microbiota functions that were altered in ABX-PREB and ABX-PREB-FMT rats; 4) FMT shifted the gut microbiota composition, diversity and predicted function in the presence of prebiotics in rats previously treated with antibiotics; 5) Manipulation of the microbiota had minimal effects on breathing, metabolism and ventilatory responsiveness to chemostimulation.

It is well-established that antibiotic administration in rodents and humans perturbs gut microbiota structure and diversity (Hoban et al., 2016; Suez et al., 2018; O'Connor et al., 2019). In our study, 8 weeks of antibiotics almost completely depleted 16S bacterial DNA (data not presented). After 4 weeks of antibiotic administration, a variety of approaches were used in our study in an effort to recolonise the gut microbiota. These included natural recolonisation, and prebiotic administration with and without FMT. We found that natural recolonisation appears the optimal approach compared with other intervention strategies (i.e. prebiotic administration with and without FMT) to normalise the gut microbiota configuration of antibiotic-treated rats; animals treated with antibiotics followed by natural recolonisation (ABX-NAT) had similar composition, diversity and function to that of vehicle treatment rats (VEH-VEH). Other investigators have reported similar findings when the gut microbiota is assessed before antibiotic treatment and after natural recolonisation. For example, in mice, 4 weeks of spontaneous recovery (natural recolonisation) following antibiotic administration partially restored baseline bacterial richness and load (Suez et al., 2018). In humans, 4 weeks after completion of ciprofloxacin administration, which has milder effects on the gut microbiota compared with the cocktail of antibiotics used in this current study, the composition of the microbiota closely resembled its state prior to antibiotic treatment (Dethlefsen et al., 2008). Furthermore, within 60 days of stopping an antibiotic treatment in humans there was 89% gut microbiota similarity to that of pre-treatment (De La Cochetiere et al., 2005). Noteworthy, in our study we did not assess the gut microbiota pre-antibiotic treatment.

Our group along with others have previously reported that prebiotic administration alters the gut microbiota composition and modulated metagenomic functions (Gibson et al., 2004; Boehme et al., 2019; O'Connor KM, 2020). Similarly, in the current study, we showed that prebiotic administration in vehicle treated rats significantly altered microbiota composition and modulated predicted function of the gut microbiota, although differences between GBMs and GMMs were not statistically significant. Probiotics have widely been used for prevention of antibiotics-associated gut microbiota disturbances and related adverse effects, although certain discrepancies are evident in terms of beneficial effects (Kechagia et al., 2013; Suez et al., 2018; Forssten et al., 2020). Although, prebiotics are also shown to have positive effects on gut microbiota composition, few studies have investigated these effects following antibiotic administration (Johnson et al., 2015; Burokas et al., 2017). We revealed that prebiotics alter gut microbiota composition and diversity in specific patterns depending on previous vehicle or antibiotic administration, with an interaction evident between ABX and prebiotics in both taxonomy and functionality. Specifically, antibiotic treated animals that received prebiotic administration thereafter (ABX-PREB and ABX-PREB-FMT rats) had increased *Blautia* relative abundance; the efficiency with which *Blautia* was able to colonise the murine gut was dependent on prior perturbation by antibiotics. This finding was accompanied by inferred functional changes in a similar direction, increasing the likelihood that these alterations have noticeable impact. Similarly, others have described that in humans, antibiotics and dietary fibres interact in their effect on metabolic function and gut microbiota composition, stating that in some cases, prebiotics restored microbiota growth and metabolic function diminished by antibiotic administration and in other cases antibiotics treatment negated the effects of the dietary fibres (Johnson et al., 2015). Interestingly, in a human study, inulin prebiotic administration resulted in elevated *Blautia* taxonomy frequency in individuals that had received gentamin antibiotic administration (Johnson et al., 2015). Our findings suggest that microbial colonisation is dependent on at least two different factors. First, as antibiotic perturbation was necessary for efficient colonisation by *Blautia*, the stability of the existing microbiome seems to be an important factor. Second, as the administration of prebiotics, GOS and FOS, were required for colonisation, the metabolic

environment of the microbiome seems to be of equal importance. Thus, it may well be that the availability of the exogenous microbe in the environment is required, however given our experimental design we did not investigate this hypothesis. Notably, as ABX-PREB and ABX-PREB-FMT cages received bedding from VEH-PREB and VEH cages, respectively this may have enhanced availability of *Blautia* in the environment (Johnson et al., 2015).

Studies have described that within 8 days of FMT in mice, alpha diversity was indistinguishable to the control group and after 28 days, composition was similar (Manichanh et al., 2010; O'Connor et al., 2019). Furthermore, autologous FMT in humans resulted in a microbiota structure similar to that of the pre-antibiotic state as early as 1 day post autologous FMT (Suez et al., 2018). On the other hand, our group and others have previously shown that FMT can disturb the gut configuration and a unique bacterial composition and diversity develops (Halkjaer et al., 2018; O'Connor et al., 2019). In the current study, we explored a combination of FMT and prebiotics to examine the effects of two well-known microbiota intervention strategies on the gut microbiota structure. Others have previously reported that FMT combined with dietary fibres showed increased short- and long-term clinical efficacy in patients with slow transit constipation (Ge et al., 2016; Zhang et al., 2018). Novel findings in the current study revealed that ABX-PREB-FMT rats had increased gut microbiota diversity, altered composition and predicted functionality compared with vehicle treated rats.

Noteworthy, ABX-PREB rats displayed a watery faecal content for 6-7 days after completing antibiotics and commencing prebiotic supplementation, whereas the ABX-PREB-FMT rats had diarrhoea-like faecal matter for 3-4 days. It is likely that prebiotic administration following antibiotic administration resulted in watery faecal content, due to the fact that there was little bacterial abundance after ABX administration, as evident by 16S bacterial DNA, to feed on dietary fibres. The influx of bacteria due to the FMT in ABX-PREB-FMT rats likely improved the diarrhoea-like

symptoms. Indeed, multiple bacteria taxa administered to the host using FMT may have thrived in the environment induced by prebiotic administration, which might contribute to important functionality in ABX-PREB-FMT rats i.e. some functional changes may be deemed beneficial such as elevations in butyrate, propionate and acetate synthesis (Roberfroid et al., 2010; Ganesh et al., 2018). On the contrary, FMT influx might result in colonisation of some pathogens that otherwise would not have been able to expand in the population. Considering the significant differences in composition, diversity and function in the ABX-PREB-FMT group, our study supports the suggestion that for successful FMT administration, pre-screening of faeces to prevent induction of pathogenic microbiota is likely required (Papanicolas et al., 2020; Stallmach et al., 2020). Notably, the FMTs were not autologous, but rather pooled from control animals. It is possible unpooled or autologous FMT would result in a different outcome in terms of recolonisation. More research is needed in this regard.

3.3.6 Summary and conclusion

Natural recolonisation is the most effective method to normalise the gut microbiota composition, diversity and predicted microbial function following antibiotic administration, which may have relevance in rodent studies assessing the role of the gut microbiota in microbiota-gut-brain axis signalling and in clinical settings, where normalisation or restoration of the gut microbiota structure is deemed a successful end-point of a therapeutic strategy. Furthermore, considering that expansion of *Blautia* is evident as a result of an antibiotic/prebiotic interaction, depending on the specific goal of a microbial intervention strategy, destabilisation of the gut microbiota might be warranted in order to ensure optimal engraftment of target microbes (Ji et al., 2017). Although parameters for engraftment and colonisation of gut microbiota are still unclear, we present evidence supporting the idea that stability of the gut microbiota may play a fundamental role in the engraftment of microbial species.

3.4 Enduring behavioral effects induced by birth by caesarean section in the mouse

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3.4.1 Summary

Birth by Caesarean (C)-section impacts early gut microbiota colonization and is associated with an increased risk of developing immune & metabolic disorders. Moreover, alterations of the microbiome have been shown to affect neurodevelopmental trajectories. However, the long-term effects of C-section on neurobehavioural processes remain unknown. Here, we demonstrated that birth by C-section results in marked but transient changes in microbiome composition in the mouse, and in particular the abundance of *Bifidobacterium spp.* was depleted in early life. Mice born by C-section had enduring social, cognitive and anxiety behavioural deficits in early-life and adulthood. Interestingly, we found that these specific behavioural alterations induced by the mode of birth were also partially corrected by co-housing with vaginally born mice. Finally, we show that supplementation from birth with a *Bifidobacterium breve* strain, or with a dietary prebiotic mixture that stimulates the growth of bifidobacteria, reverses selective behavioural alterations in C-section mice. Taken together, our data link the gut microbiota to behavioural alterations in C-section born mice and suggest the possibility of developing adjunctive microbiota-targeted therapies which may help to avert long-term negative consequences on behaviour associated with C-section birth mode.

3.4.2 Introduction

The gut microbiota -the collection of *bacteria*, archaea and eukarya residing in the gastrointestinal tract- has co-evolved with their hosts over thousands of years resulting in an intricate mutual relationship wielding significantly benefit to host health (Kundu et al., 2017). Interactions between the gut microbiota and the host involve signalling via chemical neurotransmitters and metabolites, neuronal pathways, and the immune system (Cryan et al., 2019). There is growing appreciation that microbiota, especially in early-life, influences the development and function of multiple hosts physiological systems, including the central nervous system (Sampson et al., 2016; Codagnone et al., 2019). Thus, it has been posited to be a key pillar in understanding the developmental origins of mental health and disease (Dinan and

Cryan, 2017; Codagnone et al., 2019). Preclinical studies using mice born and raised without exposure to microorganisms, germ-free mice, have highlighted the long-lasting effects of the disruption of the normal acquisition and maturation of the gut microbiota on cognition (Gareau et al., 2011), social behaviour (Desbonnet et al., 2014), and brain development (Heijtz et al., 2011). However, germ-free animals are specialized model systems and it is unclear if more medically relevant alterations in microbiome composition in early life can have enduring psychological and neurobehavioral effects.

In mammals, the composition of the gut microbiota starts to develop mainly upon birth and continues to mature and change throughout life, influenced by several factors including breast-feeding patterns (Pannaraj et al., 2017), diet (Zmora et al., 2019) antibiotic exposure (Becattini et al., 2016), and birth mode (Dominguez-Bello et al., 2010). In humans, birth by Caesarean (C)-section results in a different pattern of microbiota colonization and it is associated with increased likelihood of developing immune and metabolic disorders in childhood (Horta et al., 2013; Martinez et al., 2017; Wampach et al., 2018; Shao et al., 2019). Moreover, babies born by C-section exhibit lower relative abundance of maternally transmitted commensal bacteria and higher relative abundance of opportunistic microorganisms that are commonly found in the hospital environment (Shao et al., 2019). Despite this, the number of infants delivered by C-section worldwide has rapidly increased over recent years, and in many jurisdictions far exceeds the World Health Organization guidelines of between 10-15% (Dominguez-Bello et al., 2016). Until recently, there has been limited epidemiological data examining behavioural and psychiatric outcomes in individuals born by C-section. Associations have been made with autism, psychosis, depression, attention deficit disorder and school performance (Curran et al., 2015; O'Neill et al., 2016; Curran et al., 2017; Yang et al., 2019), though some of these associations fail to stand up when familial confounding is considered (Curran et al., 2015; O'Neill et al., 2016). Although the importance of maternal vaginal microbiome transmission for programming of the offspring brain has been recently demonstrated (Jasarevic et al., 2018), C-section-induced changes in the microbiome have been largely neglected in the context of brain health.

Within the gut microbiota bifidobacteria are among the earliest and most abundant bacterial colonizers of the gut and are essential for appropriate immune, metabolic and gastrointestinal development in infancy (Arbolea et al., 2016; Vatanen et al., 2018). The establishment of *Bifidobacterium spp.* seeding in the neonatal gut is largely influenced by vertical transmission from mother-to-infant during vaginal delivery (Hill et al., 2017; Wang et al., 2020). Birth by C-section circumvents early bifidobacterial colonization and, compared to vaginally born babies, C-section babies have decreased *Bifidobacterium spp.* relative abundance in their gut microbiota (Dominguez-Bello et al., 2010; Dominguez-Bello et al., 2016; Korpela et al., 2018; Shao et al., 2019). Although this difference tends to normalize somewhere between 6 months and 4 years (Dominguez-Bello et al., 2016; Fouhy et al., 2019), it may lead to maladaptive programming of brain and behaviour. Intervention strategies that promote a healthy balance of the gut microbiota in babies born by C-section have included the use of prebiotics and probiotics to promote growth of *Bifidobacterium spp.* and other beneficial bacteria (Moya-Perez et al., 2017).

Given the importance that initial colonization of the gut microbiota has on brain development we used a mouse model to assess the long-term consequences of birth by C-section on neurobehavioural outcomes and the potential role of gut microbiota-based interventions in remediating such effects. To interrogate these interactions, we used three different approaches. First, we compared the gut microbiota composition and neurobehavior of pups delivered by C-section and given to foster dams (C-section, CS) with pups delivered spontaneously and nursed by their own mothers (Vaginally born, VB) or by a fostered dam (Cross-fostered, CF) (**Figure 3.4-1A**). To prove the importance of the microbiome in mediating such effect we transferred microbiota from VB to CS born mice at weaning through co-housing. Co-housing may be the simplest and most convenient technique for microbial transfer as it offers opportunities of microbiota mixing between co-housed partners due to the coprophagic nature of mice (Robertson et al., 2019). Finally, we treated pups from birth with a *Bifidobacterium breve* strain, or with a dietary prebiotic mixture, which

stimulates the growth of bifidobacteria, to investigate if it could avert the long-term negative consequences on behaviour associated with delivery C-section.

3.4.3 Results

3.4.3.1 Gut microbiota alterations induced by C-section mode of birth across lifespan

To address our hypothesis that birth by C-section can affect the programming of the microbiota-gut-brain-behaviour axis, we used 16S rRNA gene sequencing to profile the gut microbiota composition in CS, VB and CF offspring in early-life (postnatal day (P) 9), pre-weaning adolescence (P21) and adulthood (week (wk) 20).

Regardless of the delivery mode, the composition of the gut microbiota was the most diverse, with regard to alpha diversity, and exhibited the highest inter-animal variability in early-life (P9), with the overall dominance of the *Lactobacillus* genus from the Firmicutes phylum (**Table S1 and Figure S1A**). Principal component (PCA) and canonical correspondence (CCA) analyses showed that the structure of the intestinal microbial community was significantly altered in both CS and CF offspring across the lifespan (**Figure 3.4-1B-D** and see also **Table S1**). Indeed, CS clustered separately from the VB and CF groups at P9, and the separation persisted throughout adolescence and adulthood (**Table S1**). From weaning onwards, the microbiota successfully re-shaped towards an approximately equal dominance of Bacteroidetes and Firmicutes phyla (see also **Table S1 and Table S2 and Table S3**), which is typical for the adult murine microbiota (Donaldson et al., 2015).

Analysis of individual bacterial taxa abundance at the phylum, family and genus levels revealed that, albeit both the CS model of delivery and the CF procedure itself had a long-lasting impact on the gut microbiota in the affected offspring, the profile of observed changes was unique for each intervention. The latter can be illustrated by the CCA plots, with CS and CF groups diverging from the VB mice (**Figure 3.4-1B-D**).

For instance, at P9, CF offspring displayed a dramatic increase in the relative abundance of *Gammaproteobacteria* species, while CS offspring was characterized by an increase in the proportion of a few Bacteroidetes genera (*Odoribacter*, *Parabacteroides*) and a marked reduction in the *Lactobacillus* bacteria (see also **Table S1**). Similarly, at P21 and wk20, various genera from the Actinobacteria and Tenericutes phyla, as well as *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* families of the Firmicutes phylum, were differentially affected by CS and CF (see also **Table S1**). Differences in the composition of the microbiota among treatment groups were associated with alterations in the short chain fatty acid (SCFA) profile, whereby caecal levels of acetate were different among groups in adolescence, but post-hoc testing did not yield significant results. Butyrate levels were higher in adulthood in CS compared with CF, but not with VB mice (see also **Table S2**).

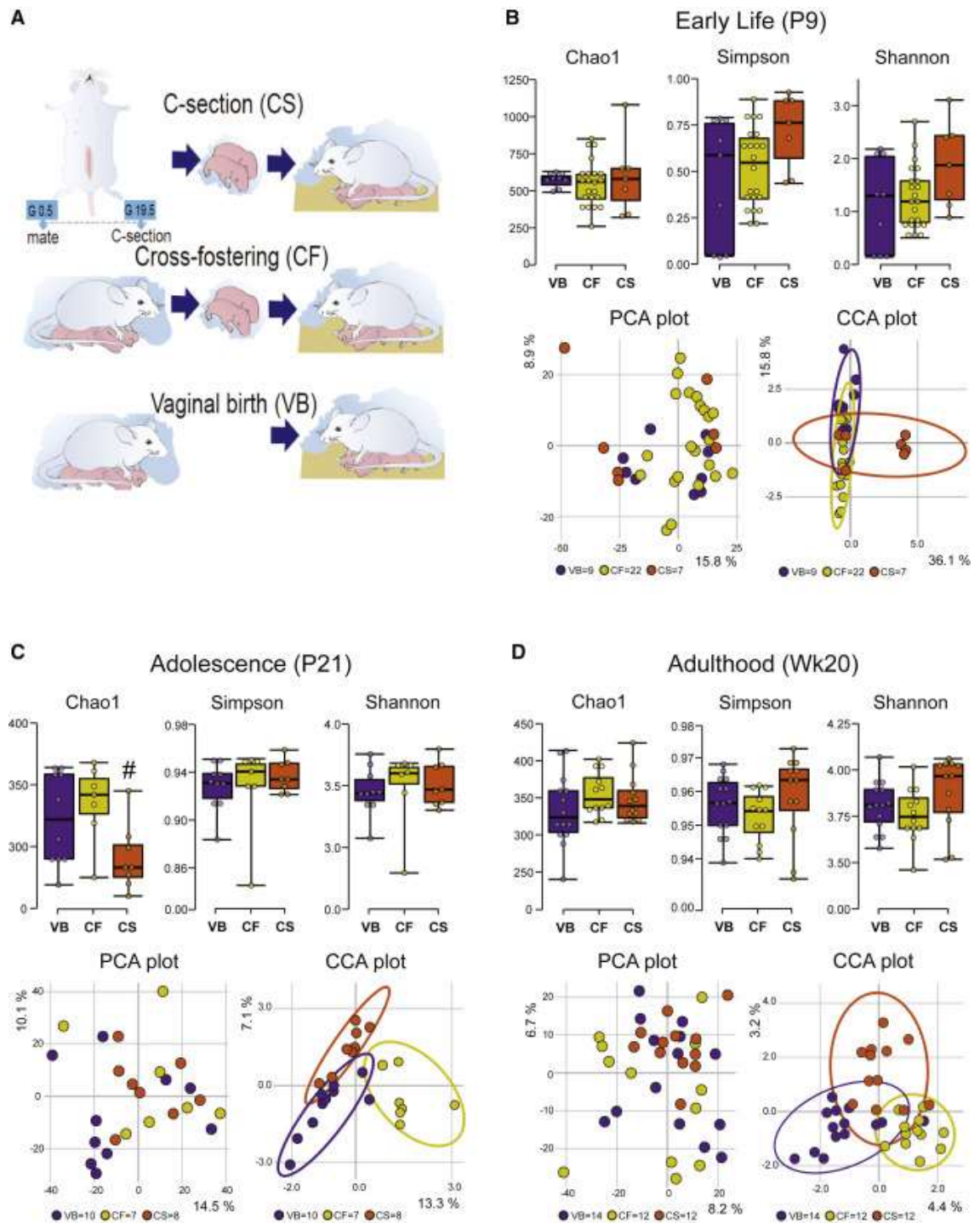


Figure 3.4-1. Mode of Delivery Affects Microbial Beta-Diversity throughout the Lifespan (A) CS animal model and experimental design. (B–D) Principal component analysis (PCA) and canonical correspondence analysis (CCA) showed that beta-diversity of intestinal (cecal) microbial community was significantly altered in the CS offspring in early-life (P9), adolescence (P21), and adulthood (week 20). CS did not impact alpha-diversity indices (Chao1, Simpson, and Shannon) at any time point. Alpha-diversity indices are presented as median and interquartile range with whiskers

representing minimum and maximum values. The x and y axes explain the variability between samples. (B) Early life (P9; VB n = 9, 4 litters; CF n = 22, 4 litters; CS n = 7, 4 litters). (C) Adolescence (P21; VB n = 10, 4 litters; CF n = 7, 4 litters; CS n = 8, 4 litters), #p < 0.05 CS versus CF. (D) Adulthood (VB n = 14, 4 litters; CF n = 12, 4 litters; CS n = 12, 4 litters).

CF, cross-fostering; CS, C-section; VB, vaginal birth. Statistical details: Among-group differences in alpha-diversity indices were analyzed with Mann-Whitney U test. Benjamini-Hochberg adjustment with $Q = 0.2$ was used to correct p values for multiple testing. PCA plots at the operational taxonomic unit (OTU) level were constructed using Aitchison distance calculated in the ALDEx2 library; PCA was done using the prcomp() function. CCA plots at the OTU level were generated with the vegan library; ellipses represent 95% confidence interval calculated by the ggplot2 library. The vegan implementation of PERMANOVA followed by PERMANOVA as a post hoc was used to test for differences at a beta-diversity level; Data S1; Figure S1. See also Data S3 and S4 and Table S1.

3.4.3.2 C-section delivery leads to neurobehavioural changes in early life

We then compared the consequences of mode of delivery on offspring behaviour in early-life, particularly focusing on social behaviour, cognitive, and anxiety-like aspects of a behavioural phenotype (**Figure 3.4-2A**). Quantification of ultrasonic vocalization (USV) is widely used to measure early communicative behaviour and aversive affective reactions to stress separation (Jung et al., 2018). Here we found that, in early life (P9), CS offspring exhibited a higher number of USV calls when isolated from their littermates and mother than CF or VB animals (**Figure 3.4-2B**). It has been previously demonstrated that by P10 pups are normally able to respond to relevant social stimuli and to efficiently discriminate their mother's nest when physically separated (Macri et al., 2010). Unlike VB or CF animals, CS pups had less preference for the maternal versus neutral bedding (**Figure 3.4-2C-D**), thus expressing early social recognition and maternal attachment deficits. Together these results suggest that birth by C-section is interfering with early-life communication, perception of relevant signals, and association with particular environmental contexts.

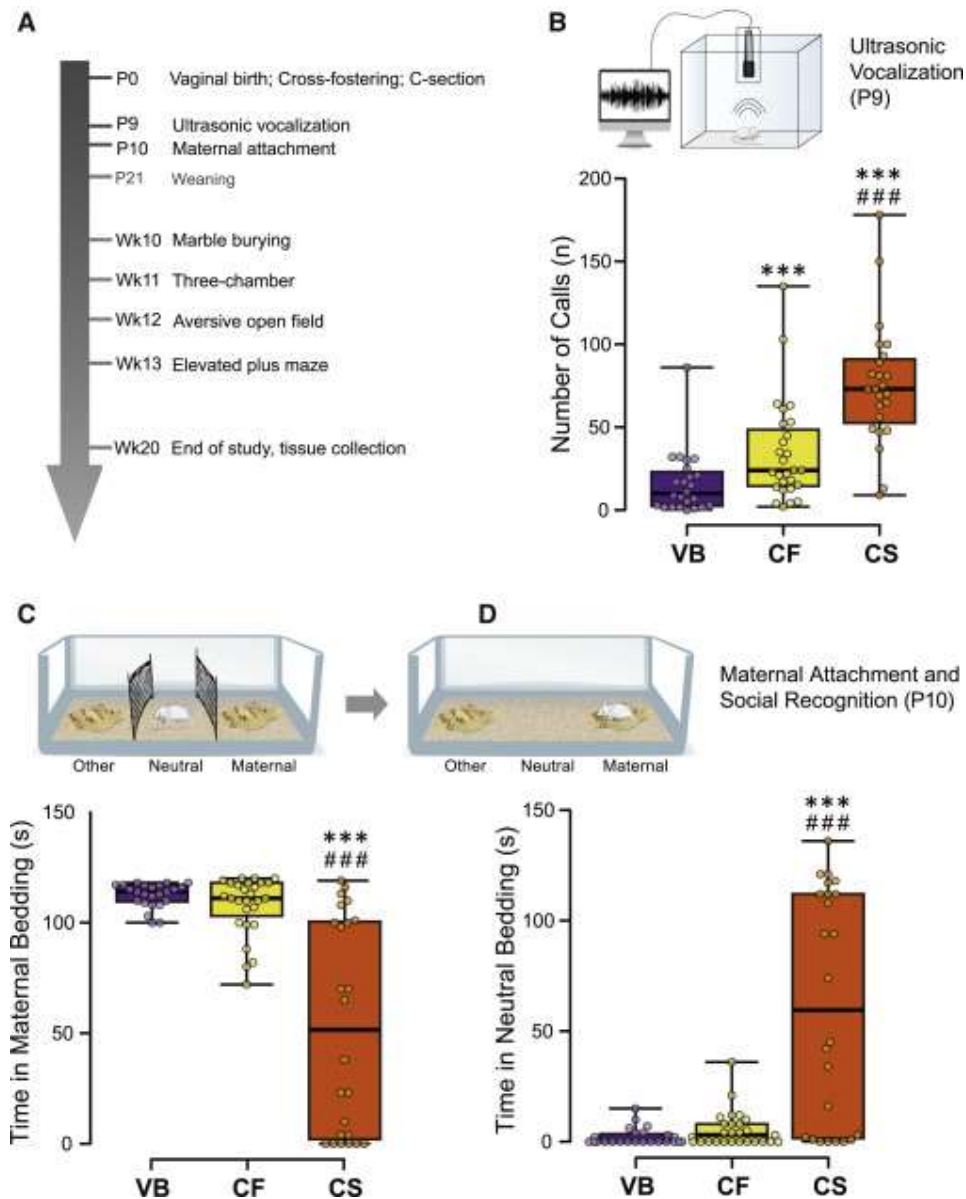


Figure 3.4-2. CS Delivery Mode Leads to Neurobehavioral Changes in Early Life (A) Experimental timeline. (B) CS-born offspring exhibited communication deficits and anxiety-like behavior at P9 as measured by increased number of USV calls. $***p < 0.0001$ CS versus VB; $###p < 0.0001$ CS versus CF. (C and D) CS-born mice exhibited deficits in maternal attachment behavior at P10. (C) CS-born offspring failed to exhibit preference for their home/maternal bedding, $***p < 0.0001$ CS versus VB; $###p < 0.0001$ CS versus CF. (D) CS-born offspring displayed increased preference for a neutral bedding; $***p < 0.0001$ CS versus VB; $###p < 0.0001$ CS versus CF. All data are presented as median and interquartile range with whiskers representing minimum and maximum values. VB $n = 24$, 4 litters; CF $n = 12$, 4 litters; CS $n = 24$, 4 litters. USV, ultrasonic vocalization. Statistical details: (B) number of calls ($x^2 = 33.303$; $p < 0.001$); (C) time spent on the home/maternal bedding ($x^2 = 26.106$; $p < 0.0001$); and (D) time spent on a neutral bedding ($x^2 = 20.577$; $p < 0.0001$). (B–D) Among-group differences

were analyzed with Kruskal-Wallis test, followed by Mann-Whitney U test. See also Data S3 and S4.

3.4.3.3 Enduring neurobehavioural effects induced by C-section

Alterations in sociability are a common feature among a variety of neuropsychiatric conditions, and microbiota-deficient mice develop social deficits (Desbonnet et al., 2014). Here we investigated whether mice born by C-section exhibit deficits in social behaviour in adulthood. Although CS mice displayed normal sociability in the three-chamber test (i.e preference for mouse over object) (**Figure 3.4-3A**), a specific deficit in social novelty recognition (i.e preference for novel over familiar social partner) was revealed in CS mice compared with VB and CF offspring (**Figure 3.4-3B**). Interestingly, during the subsequent intervention studies where we probed adult CS mice against non-social cognitive cues in the novel object recognition test, the CS mice failed to discriminate between a novel and a familiar object in active investigation time (the effect was not significant in investigation index, see also **Figure S2**).

The hippocampus is an important brain area for learning and memory as well as for the regulation of the stress response (Levone et al., 2015). Accumulating data also show that it represents a key node in the microbiome-gut-brain axis, with alterations in the gut microbiome being associated with changes in hippocampal gene expression, neurogenesis, and neurotransmission (Clarke et al., 2013; Ogbonnaya et al., 2015; Burokas et al., 2017; Chen et al., 2017). In addition, the hippocampus is required for proper social recognition (Raam et al., 2017) and social memory formation (Phillips et al., 2019). Thus, it was important to investigate whether the hippocampus transcriptome was sensitive to C-section induced changes in the gut microbiota. In agreement with the behavioural data, the transcriptome analysis of the hippocampal brain region in adult mice revealed substantial transcriptional differences in the CS offspring (**Figure 3.4-4A-B**). CS mice clustered separately from either VB or CF counterparts, while no differences between CF and VB groups were observed. Interestingly, of the 38 genes up-regulated in CS mice, nine belonged to extracellular matrix-associated group (Col8a1, Col8a2, Col4a3, Ctsc, Frdc9, Itih5, etc).

In addition to alterations in social novelty recognition in adulthood, CS mice exhibited exaggerated anxiety-like behaviour as observed by increased number of buried marbles in the marble burying test (**Figure 3.4-3C**), reduced number of entries into the open arms in the EPM test (**Figure 3.4-3D-E**), and reduced locomotion and time spent in the central zone of the OF test (**Figure 3.4-3F-G**). Most of the CS-associated effects on anxiety remained significant after adjustment for the litter effect (see also **Table S3**), but failed to be robustly evident in subsequent cohorts (see also **Figure S3**). This suggests a subtle nature of the pro-anxious behavioural phenotype in the CS offspring, and/or the importance of postnatal environment for the development of these outcomes. In contrast, CS-induced deficits in social novelty recognition not only withstood the adjustment for the litter effect (see also **Table S3**), but were consistently observed across all experimental cohorts (**Figure 3.4-5C** and **Figure 3.4-6H**), thus indicating the robustness of the observed effects. Controlling for the early environment exposure was an important goal of the initial experiments and that is why for this first set of experiments we included all three groups (CS, CF and VB), that would give us a fully balanced stratified experimental design. Although the CF procedure itself resulted in a unique effect on the gut microbiota (**Figure 3.4-1 B-D**), these changes did not manifest in many behavioural alterations throughout (**Figure 3.4-2** and **Figure 3.4-3**). For statistical, logistical and ethical reasons (in order to meet 3R requirements and minimize animal usage), we chose to only have VB group as control in the follow-up studies.

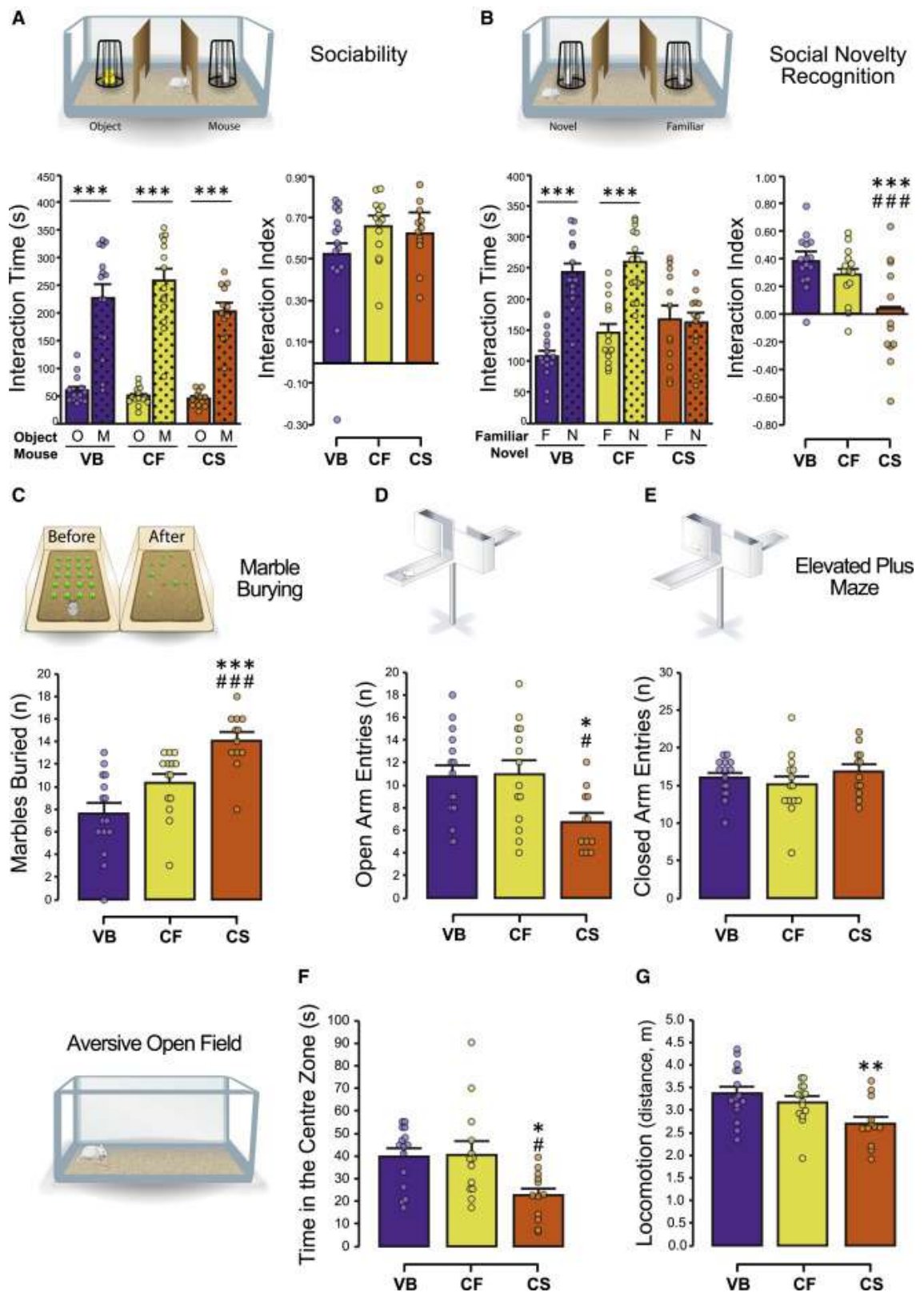


Figure 3.4-3. Enduring Neurobehavioral Effects Induced by CS (A and B) CS delivery mode had an impact on social behavior in adulthood (three-chamber test).

(A) CS did not impair sociability. $***p < 0.001$ mouse versus object for the interaction time data. (B) CS-born mice had deficits in social novelty recognition. $***p < 0.001$ novel versus familiar mouse for the interaction time data. $***p < 0.001$ CS versus VB and $^{###}p < 0.001$ CS versus CF for the interaction index data. (C–G) In adulthood, mice delivered by CS displayed enhanced anxiety-like behavior across various tests. (C) Increased number of buried marbles in the CS group. $***p < 0.001$ CS versus VB and $^{###}p < 0.001$ CS versus CF. (D) Decreased number of entrances into the open arms in the CS group. $*p < 0.05$ CS versus VB and $^{\#}p < 0.05$ CS versus CF. (E) Number of entrances in the closed arms were unchanged. (F) Reduced time spent in the center zone of an aversive open-field arena in the CS group $*p < 0.05$ CS versus VB; $^{\#}p < 0.05$ CS versus CF. VB $n = 15$, 4 litters; CF $n = 13$, 4 litters; CS $n = 12$, 4 litters. (G) Reduced total distance traveled in the aversive open field in the CS group. $**p < 0.05$ CS versus VB. Data are presented as mean + standard error of the mean (SEM). (A–E and G) VB $n = 15$, 4 litters; CF $n = 14$, 4 litters; CS $n = 12$, 4 litters. Statistical details: (A) interaction time: VB $t(14) = 6.341$, $p < 0.0001$; CF $t(13) = 9.776$, $p < 0.0001$; CS $t(11) = 9.811$, $p < 0.0001$, paired Student's t test. Interaction index: $F(2,38) = 1.555$; $p = 0.224$; one-way ANOVA followed by Tukey post hoc tests. (B) Interaction time: VB $t(14) = 7.8$; $p < 0.001$; CF $t(13) = 5.1$; $p < 0.0002$; CS $t(11) = -0.167$; $p = 0.8707$; paired Student's t test. Interaction index: $F(2,38) = 14.73$; $p < 0.0001$; one-way ANOVA followed by Tukey post hoc tests. (C) Marbles: $F(2,38) = 14.73$; $p < 0.0001$. (D) Entrances to open arms: $F(2,38) = 4.74$; $p = 0.015$. (E) Entrances to closed arms: $F(2,38) = 0.614$; $p = 0.4047$. (F) Time in the center zone: $F(2,37) = 1.077$; $p = 0.0076$. (G) Distance: $F(2,38) = 5.22$; $p = 0.01$. (C–G) One-way ANOVA, followed by Tukey post hoc tests. See also [Data S3](#) and [S4](#) and [Table S2](#).

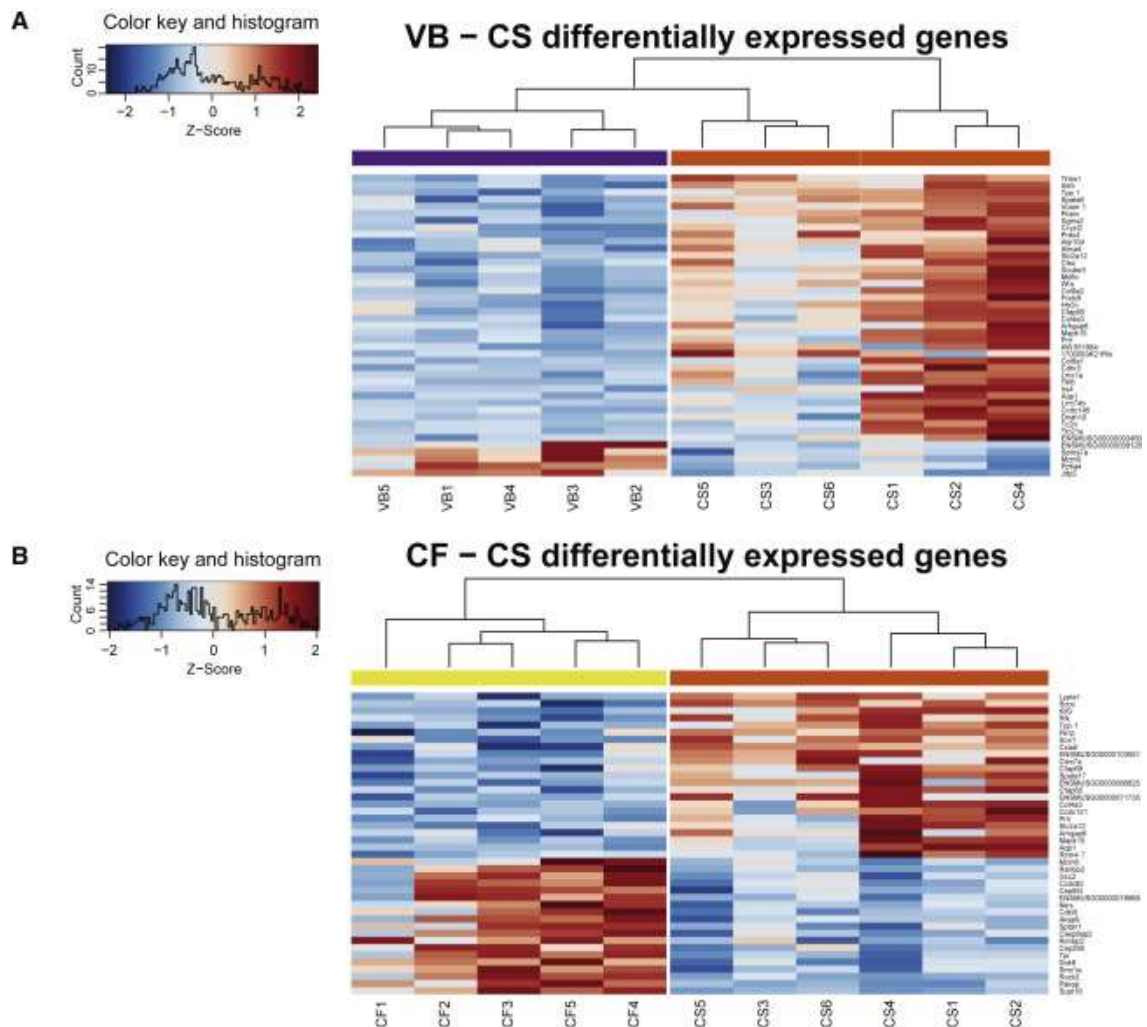


Figure 3.4-4. CS Mode of Birth Induces Enduring Changes in the Hippocampal Transcriptome Heatmap showing differentially expressed genes in the adult hippocampus of CS versus VB offspring (A) and CS versus CF offspring (B). Differential gene expression was determined using the DESeq2 R-package (v1.6.2) with default parameters on pairwise comparisons of all possible group combinations. An adjusted $p \leq 0.1$ (Benjamini-Hochberg method) was considered statistically significant. Red color indicates increased expression, and blue color indicates decreased expression levels of the affected genes. VB $n = 5$, 4 litters; CF $n = 5$, 4 litters; CS $n = 6$, 4 litters. See also [Data S3](#) and [S4](#).

3.4.3.4 Microbiota transfer by co-housing reverses specific neurobehavioural changes induced by C-section

To investigate a potential causal role for the gut microbiota in mediating the observed behavioural changes, we examined whether transferring microbiota from VB to CS born mice at weaning could prevent C-section-mediated behavioural

deficits. We exploited the coprophagic nature of mice and performed faecal transfer by co-housing one CS mouse with three VB mice in adolescence (based on the strategy utilized by Buffington and colleagues, (Buffington et al., 2016)). Littermates originating from multiple litters were randomly assigned to the different housing systems to minimize the litter effect. Behaviour was assessed in adulthood (**Figure 3.4-5A**). Although CS mice displayed normal sociability in the three-chamber test (i.e mouse vs object) (**Figure 3.4-5B**), co-housing CS with VB mice selectively reversed C-section-induced cognitive deficits, restoring social novelty recognition (**Figure 3.4-5C**). Despite not affecting the marble-burying or EPM readouts (see also **Figure S5B-C**), co-housing had anxiolytic effects in CS mice, increasing the time spent in the central zone of the OF (see also **Figure S5D**).

Next, we investigated the gut microbiota composition in VB, CS and CS co-housed offspring at week 4, i.e one week following the commencement of the co-housing regimen). Co-housing did not affect alpha-diversity indices (see also **Figure S4A, Table S4**). Moreover, the PCA analysis did not show significant differences in the microbial communities structure across groups (**Figure 3.4-4D**), though all three groups clustered separately on the CCA plot (**Figure S4B**, $p < 0.05$, PERMANOVA, Table S4, beta diversity analysis). When we looked at the individual bacterial taxa that showed the strongest response to mode of delivery or co-housing regimen, we observed that co-housing reversed C-section-associated reduction in the *Bacteroidetes* genus (**Figure 3.4-5E**). Furthermore, co-housing had a unique effect on the relative abundance of *Blautia* and *Rikenella* bacteria, while not affecting *Bacteroidales* S24-7 group and *Anaeroplasma* species in CS mice (**Figure 3.4-5E**). These data support the concept of plasticity within the microbiome-gut-brain axis and show that the enduring effects of C-section can be at least partially restored via microbial transfer.

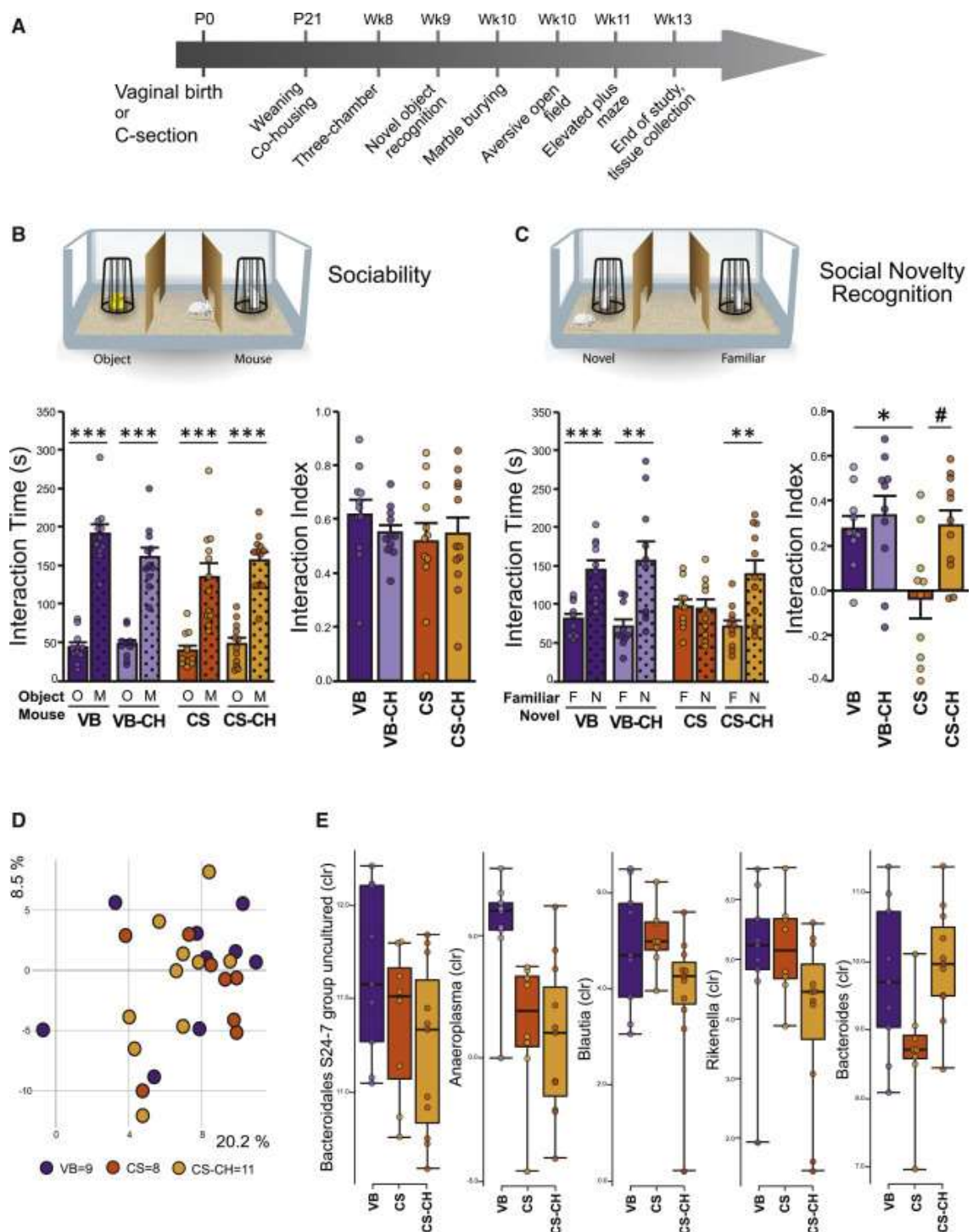


Figure 3.4-5. Microbiota Transfer by Co-housing Partially Restores CS Behavioral Phenotype (A) Experimental timeline of the co-housing study. (B) Co-housing did not affect sociability; *** $p < 0.001$ for mouse versus object for the interaction time data. VB $n = 11$, 9 litters; CS $n = 12$, 6 litters; VB-CH, $n = 11$, 9 litters; and CS-CH, $n = 12$, 7 litters. (C) Co-housing reversed social novelty recognition deficits in CS-born mice; ** $p < 0.01$ and *** $p < 0.001$ for novel versus familiar mouse for the interaction time data. * $p < 0.05$ CS versus VB and # $p < 0.05$

CS versus CS-CH for the interaction index data. Social novelty: VB $n = 10$, 9 litters; CS $n = 10$, 6 litters; VB-CH, $n = 10$, 9 litters; and CS-CH, $n = 11$, 7 litters. (D) PCA did not show significant differences in the beta diversity among all groups in the intestinal (fecal) microbiome community (see also [Data S2](#)). The x and y axes explain the variability between samples. (E) Co-housing restored CS-associated reduction in the Bacteroidetes genus. Relative abundance of the bacterial taxa (clr) with the strongest response to mode of delivery and/or housing regimen. Data are presented as mean + SEM on (B) and (C) and as median and interquartile range with whiskers representing minimum and maximum values (E). (D and E) VB $n = 9$, 9 litters; CS $n = 8$, 6 litters; and CS-CH, $n = 11$, 7 litters. CS-CH, CS co-housed. Statistical details: (B) interaction time: VB $t(10) = 8.863$, $p = 0.001$; VB co-housed $t(10) = 11.94$, $p = 0.0001$; CS $t(11) = 4.920$, $p = 0.0005$; CS co-housed $t(11) = 8.835$, $p < 0.0001$, paired Student's t test. Interaction index: group effect $F(1,42) = 0.146$, $p = 0.705$; mode of delivery effect $F(1, 42) = 0.557$, $p = 0.646$; group \times mode of delivery effect $F(1,42) = 0.692$, $p = 0.410$, two-way ANOVA followed by Tukey post hoc. (C) Interaction time: VB $t(9) = 4.566$, $p = 0.001$; VB co-housed $t(8) = 2.902$, $p = 0.0198$; CS $t(9) = 0.7423$, $p = 0.7873$; CS co-housed $t(10) = 4.133$, $p = 0.002$, paired Student's t test. Interaction index: group effect $F(1,37) = 6.49$, $p = 0.0151$; mode of delivery effect $F(1,37) = 5.565$, $p = 0.0237$; group \times mode of delivery effect $F(1,37) = 3.203$, $p = 0.0817$, two-way ANOVA, followed by Tukey post hoc. (D) Beta-diversity, PCA plots, pairwise PERMANOVA, $p < 0.001$, [Data S2](#) and [Figures S2–S4](#). See also [Data S3](#) and [S4](#) and [Table S2](#).

3.4.3.5 *Bifidobacterium* spp. contribute to C-section-induced neurobehavioural changes

Since 16S rRNA gene sequencing provides a general overview of microbial community structure, we next employed a quantitative reverse transcription polymerase chain reaction (RT-qPCR) approach to look at the absolute abundance of specific bacterial taxa. We focused on the *Bifidobacterium* genus, since mode of delivery was shown to be an important factor in shaping bifidobacteria colonization in infants (Penders et al., 2006; Dominguez-Bello et al., 2010; Fouhy et al., 2019). We quantified *Bifidobacterium* species in the faeces of VB and CS mice at weaning (wk 3), adolescence (wk 4) and in adulthood (wk 7). Herein, we demonstrate a transient significant decrease in *Bifidobacterium* spp. abundance in CS offspring at weaning (wk 3), which was no longer observable one week or four weeks later (**Figure 3.4-6A**). Given the fact that bifidobacteria are among the earliest bacterial colonizers of the neonatal gut and are essential for appropriate immune, metabolic and gastrointestinal development in infancy, disturbances in their appropriate

establishment at the beginning of life could have long-term neurobehavioural effects. To this end, we used two different methods of dietary intervention to augment *Bifidobacterium* levels in our mouse model (**Figure 3.4-6B**). We supplemented CS-nursing dams through their diet with either human commensal *Bifidobacterium breve* M16-V (*B. breve*) or a prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) in a 9:1 ratio, known to promote *Bifidobacterium* growth (Kosuwon et al., 2018). At wk 3, CS pups were weaned onto the corresponding maternal diet. Both scGOS/lcFOS and *B. breve* supplementation successfully restored early-life deficit in the *Bifidobacterium* spp. abundance associated with CS (**Figure 3.4-6C**). Notably, even as early as at P9, treatment with the prebiotic mixture prevented communication deficits by reducing the number of USV calls emitted by the CS pups when they were isolated from their nest (**Figure 3.4-6D**). Moreover, at P10, both interventions successfully restored neonatal recognition abilities and maternal attachment deficits in the CS pups (**Figure 3.4-6E-F**). As in **Figure 3.4-3**, social and non-social recognition, as well as anxiety-like behaviour were assessed in adulthood (**Figure 3.4-6G-H**, see also **Figure S3**). In adulthood, CS-induced social recognition impairment persisted in mice treated with *B. breve*, while treatment with scGOS/lcFOS completely reversed this deficit (**Figure 3.4-6H**). Moreover, scGOS/lcFOS treatment restored novel object recognition deficits (see also **Figure S4C-D**) in the CS group, with all positive cognitive effects remaining significant after controlling for postnatal environment litter effect.

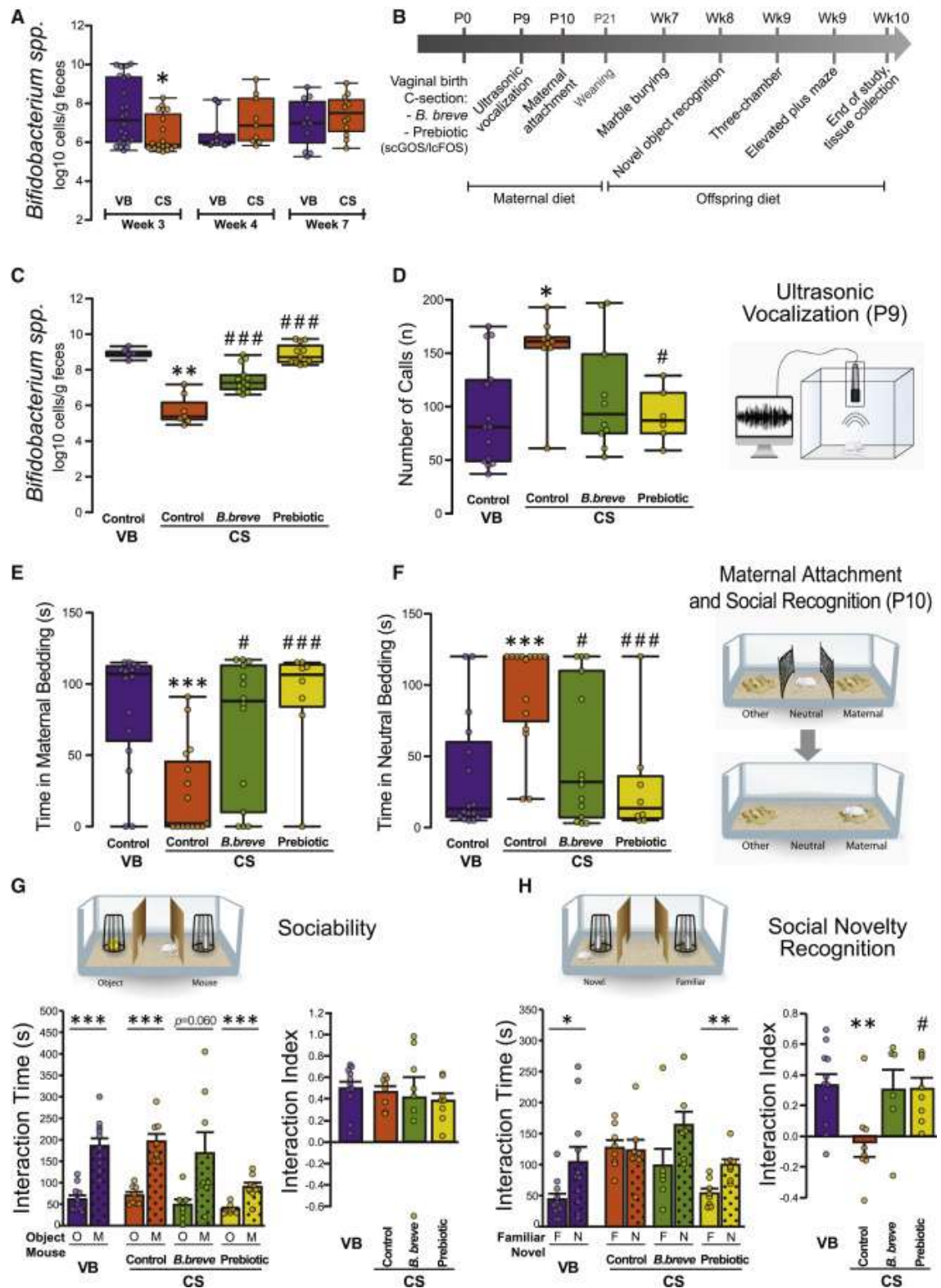


Figure 3.4-6. Targeting *Bifidobacterium* Genus from Birth Restores Behavioral Deficits in CS Mice (A) Transient significant decrease in *Bifidobacterium* spp. abundance (log₁₀ cell/g feces) was seen in the CS offspring at weaning (week 3). Week 3 VB n = 24, 9 litters; CS n = 19, 6 litters; week 4 VB n = 9, 9 litters; CS n = 11, 6

litters; and week 7 VB $n = 7$, 4 litters; CS $n = 6$, 7 litters. $*p < 0.05$ CS versus VB. (B) *B. breve* and scGOS/lcFOS administration and experimental timeline. (C) Treatment with scGOS/lcFOS and *B. breve* restored early-life deficit in the *Bifidobacterium* spp. abundance (\log_{10} cell/g feces) associated with CS. $**p < 0.01$ CS versus VB; $###p < 0.001$ CS versus CS+B. *breve* and CS versus CS+prebiotic. VB $n = 5$, 4 litters; CS $n = 7$, 3 litters; CS+B. *breve*, $n = 13$, 3 litters; and CS+prebiotic, $n = 11$, 3 litters. (D) Prebiotic mixture attenuated communication deficits in CS-born mice at P9; *B. breve* supplementation had no effect on early-life communication and anxiety. $*p < 0.05$ CS versus VB; $\#p < 0.05$ CS versus CS+prebiotic. VB $n = 14$, 4 litters; CS $n = 9$, 3 litters; CS+B. *breve*, $n = 10$, 3 litters; and CS+prebiotic, $n = 6$, 3 litters. (E and F) scGOS/lcFOS and *B. breve* treatments restored maternal attachment deficits in the CS pups at P10. VB $n = 16$, 4 litters; CS $n = 15$, 3 litters; CS+B. *breve*, $n = 14$, 3 litters; and CS+prebiotic, $n = 8$, 3 litters. (E) Time spent on the maternal bedding. $***p < 0.001$ CS versus VB; $\#p < 0.05$ CS versus CS+B. *breve*; $###p < 0.05$ CS versus CS+prebiotic. (F) Time spent on the neutral bedding $***p < 0.001$ CS versus VB; $\#p < 0.05$ CS versus CS+B. *breve*; $###p < 0.05$ CS versus CS+prebiotic. (A–F) Data are presented as median and interquartile range with whiskers representing minimum and maximum values. (G) Treatment with prebiotic did not affect sociability. $***p < 0.001$ for mouse versus object for the interaction time data. VB $n = 11$, 4 litters; CS $n = 8$, 3 litters; CS+B. *breve*, $n = 8$, 3 litters; and CS+prebiotic, $n = 8$, 3 litters. (H) Treatment with prebiotic reversed social novelty recognition deficits in CS-born mice. *B. breve* supplementation had no effect on social novelty recognition. $*p < 0.05$ and $**p < 0.01$ for novel versus familiar mouse for the interaction time data. $*p < 0.01$ CS versus VB and $p < 0.05$ CS versus CS+prebiotic for the interaction index data. VB $n = 11$, 4 litters; CS $n = 8$, 3 litters; CS+B. *breve*, $n = 6$, 3 litters; and CS+prebiotic, $n = 8$, 3 litters. (G and H) Treatment with prebiotic did not affect sociability but reversed social novelty recognition deficits in CS-born mice. *B. breve* supplementation had no effect on social novelty recognition. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ for mouse versus object and novel versus familiar mouse for the interaction time data. $*p < 0.01$ CS versus VB and $p < 0.05$ CS versus CS+prebiotic for the interaction index data. Data are presented as mean + SEM. Statistical details: (A) week 3, $U = 129.00$, $p = 0.015$; week 4, $U = 56.5$, $p = 0.161$; and week 7, $U = 18.00$, $p = 0.070$, Mann-Whitney U test. (C) *Bifidobacterium* spp. abundance: CS versus VB, $\chi^2 = 0.000$, $p = 0.004$, Mann-Whitney U test; CS versus CS+treatment, $\chi^2 = 20.472$, $p < 0.0001$, Kruskal-Wallis test followed by multiple comparisons. (D) Number of calls: CS versus VB, $U = 27.500$, $p = 0.025$, Mann-Whitney U test; CS versus CS+treatment; $\chi^2 = 6.203$, $p = 0.045$, Kruskal-Wallis test followed by multiple comparisons. (E) Time spent on the home/maternal bedding; CS versus VB, $\chi^2 = 35.000$, $p = 0.001$, Mann-Whitney U test; CS versus CS+treatment, $\chi^2 = 10.484$, $p = 0.005$, Kruskal-Wallis test followed by multiple comparisons. (F) Time spent on the neutral bedding: CS versus VB, $\chi^2 = 35.000$, $p = 0.001$, Mann-Whitney U test; CS versus CS+treatment; $\chi^2 = 10.484$, $p = 0.005$, Kruskal-Wallis test followed by multiple comparisons test. (G) Sociability. Interaction time: VB $t(10) = 6.150$, $p = 0.0001$; CS $t(7) = 6.813$, $p = 0.001$; CS+B. *breve* $t(7) = -2.236$, $p = 0.060$; CS+prebiotic $t(7) = 4.662$, $p = 0.0023$, paired Student's t test. Interaction index: CS versus VB $t(17) = 0.349$, $p = 0.731$, unpaired Student's t test; CS versus CS+treatment groups $F(2,21) = 0.1374$, $p = 0.8724$, one-way ANOVA followed by Tukey post hoc tests. (H) Social novelty recognition. Interaction time: VB $t(10) = 2.974$, $p = 0.014$; CS $t(7) = 0.1795$, $p = 0.8626$;

CS+B. breve $t(5) = 1.588$, $p = 0.1232$, *CS+prebiotic* $t(7) = 3.776$, $p = 0.0069$, paired Student's t test. Interaction index: *CS* versus *VB*, $t(17) = 3.053$, $p = 0.007$, unpaired Student's t test; *CS* versus *CS+treatment* groups, $F(2,21) = 4.379$, $p = 0.027$, one-way ANOVA followed by Tukey post hoc. See also Data S3 and S4, Table S2, and Figures S2 and S3.

3.4.4 Discussion

Thousands of years of interkingdom symbiosis between gut microorganisms and their animal hosts have influenced the hosts physiological systems development, including the central nervous system (Sherwin et al., 2019). Birth is one of the key factors shaping the gut microbiota structure in mammals and maternal transmission of the gut microbiota has likely contributed to the establishment of this evolutionary symbiotic relationship in many different species (Funkhouser and Bordenstein, 2013). In humans, mode of delivery at birth is one of the key factors regulating early-life gut microbiota composition of mammals (Penders et al., 2006; Martinez et al., 2017). Here, we establish a mouse model of C-section mode of delivery which recapitulates structural changes in the intestinal microbial community in early life that endured through adolescence. Previous human studies have demonstrated that C-section significantly reduces *Bifidobacterium* spp. Abundance in the infant intestine, with the observed deficit normalising later in life (Dominguez-Bello et al., 2010; Fouhy et al., 2019). In agreement, our model shows a significant and transient depletion of *Bifidobacterium* spp. in the CS offspring in early life. Altered microbiome composition at critical stages of early life, during which rapid development and maturation of central nervous system occur, has been implicated in a variety of behavioural alterations in animals (O'Mahony et al., 2017) and humans (Christian et al., 2015; Carlson et al., 2018; Cowan et al., 2020). However, until recently there has been limited epidemiological data examining behavioural and psychiatric outcomes in individuals born by C-section, and scarce data that exists from animal models was inconclusive (Vaillancourt and Boksa, 1998; El-Khodori and Boksa, 2002; Castillo-Ruiz

et al., 2018; Swift-Gallant et al., 2018; Chiesa et al., 2019; Yang et al., 2019). Here we demonstrate that structural alterations in the intestinal microbial community induced by C-section are associated with robust and persistent behavioural changes in the affected offspring. CS mice display social communication and maternal attachment deficits in early-life, and specific impairment of social novelty recognition in adulthood. CS-induced deficits in recognition also extend to discrimination of non-social cognitive cues.

In order to establish if disturbances in the appropriate colonization of bifidobacteria at the beginning of life is implicated in the observed behavioural deficits, we used two alternative approaches to counteract the reduction in *Bifidobacterium* spp. abundance induced by C-section (dietary supplementation of either *B. breve* strain or a prebiotic mixture of scGOS/lcFOS). Treatment with both strategies successfully reversed social and non-social recognition deficits in the CS offspring. Thus, we provide here a causal link between deficits in early life bifidobacteria colonization of the gut and the behavioural phenotype associated with C-section. Strikingly, in a recent human study, maternal supplementation with a *B.breve* strain completely reversed the impact of birth by C-section and antibiotic treatment on the microbiota composition in infants (Chua et al., 2017).

In the co-housing experiment, we demonstrated that non-specific faecal microbiota transfer from the VB to the CS offspring at weaning was similarly effective in reversing C-section-induced behavioural deficits, and was associated with partial restoration of gut microbiota composition in the CS offspring. This further supports the implication of gut bacteria in mediating specific behavioural changes associated with C-section. Here we showed that co-housing had a unique effect on the relative abundance of *Blautia* and *Rikenella* bacteria, while not affecting *Bacteroidales* S24-7 group and *Anaeroplasma* species in CS mice. Thus, the exact bacteria involved in restoring behavioural effects are unclear and remain to be explored in future intervention studies (Sbahi and Di Palma, 2016). It should be acknowledged that

transmission of the microbiota via coprophagy may have limited efficacy on microbiota standardisation as it select for bacteria that are more tolerant of certain environments and able to conquer resident microbiota response to colonization in the recipient mouse (Robertson et al., 2019). Further, co-housing mice that express different behaviours may have an effect itself. Thus, the effects of co-housing with CS with VB may not be entirely to faecal microbiome transfer.

A growing body of work implicates the gut microbiota in social behaviour and cognitive performance, and alterations of microbiota have been recently associated with neurodevelopmental disorders (Hsiao et al., 2013; Desbonnet et al., 2014; Buffington et al., 2016). The precise mechanism by which C-section affects the developing brain and behaviour remains to be determined. However, pathways of communication that may be involved include alterations in vagus nerve signalling, immune system response, metabolite production including bile acids, tryptophan metabolism, enteroendocrine signalling, and changes in blood-brain and gastrointestinal barrier permeabilities (Cryan et al., 2019). Future studies should integrate behavioural outcomes with more functional analysis of the gut microbiota including metabolomic and metagenomic profiling which will allow for a more mechanistic view of microbiota gut-brain axis alterations in C-section. We observed differential expression of genes belonging to the extracellular matrix-associated group in the hippocampus of the CS offspring. Changes to this gene cluster have been associated with formation of memory (Tsien, 2013), cognitive flexibility (Happel et al., 2014), synaptic plasticity, and autistic-like behaviours in animal models (Jung et al., 2018). In line with our behavioural findings, C-section has been previously suggested to alter the dopaminergic system (Vaillancourt and Boksa, 1998; El-Khodori and Boksa, 2002), to increase neuronal cell death in the mouse brain and specifically affect vasopressin neurons in the hypothalamus (Swift-Gallant et al., 2018), the latter being important for social behaviour and recognition. The role of microbiota in the remodelling of these pathways has yet to be elucidated. As previously demonstrated in the extreme situation in germ-free mice, our results reinforce the importance of

gut microbiota composition in early life on normal host neurobehavioural development.

3.4.5 Conclusion

Together our findings raise significant concerns regarding the overuse of elective C-section deliveries in modern medicine because of likely consequential changes in the microbiome and neurobehavioural effects. However, it is worth noting that along with the microbiota, C-section can affect other physiological changes, such as stress and immune priming during the birthing process, all of which may also contribute to the phenotype (Lagercrantz and Slotkin, 1986). It is clear that certain keystone species (including *Bifidobacterium spp.*) are vitally important during critical windows of development; they contribute to essential immune priming and represent a viable target for dietary intervention in mothers and infants. Restoration of bifidobacteria imbalance in C-section delivered infants represents a challenge that can be addressed in many ways. Recently, partial restoration of the gut microbiota of infants born by C-section was demonstrated via vaginal microbial transfer (Dominguez-Bello et al., 2016). Vaginal seeding, performed by swabbing babies with vaginal fluid over their entire bodies, successfully colonized the newborn gut with maternal vaginal microbes for up to 30 days (Dominguez-Bello et al., 2016). It should be noted though, in cases of C-section, vaginal seeding is currently considered unsafe due to the potential transfer of pathogenic bacteria to the newborn infant (2017; Haahr et al., 2018). Dietary intervention may represent a more acceptable approach, both interventions (dietary supplementation of either *B. breve* strain or a prebiotic mixture of scGOS/lcFOS) did not interfere with the further colonization of native bifidobacteria and represent a safer alternative to vaginal seeding (Moya-Perez et al., 2017).

Our study is not without limitations; we use only male mice to allow us to compare our findings with previously published data from both our group and others on the role of the microbiome in behaviour and neurodevelopment (Jaggar et al., 2019).

Future studies should focus on interrogating the impact of C-section-induced microbiota changes on behaviour in female mice (Lagercrantz and Slotkin, 1986). Moreover, these studies now call for the investigation of the long-term impact of C-section on brain and behaviour in other mouse strains and other species including humans. Finally, since C-section deliveries when medically indicated, are unavoidable lifesaving interventions; our data point to the possibility of developing adjunctive microbiota-targeted therapies (Dominguez-Bello et al., 2016; Moya-Perez et al., 2017) in this vulnerable population. Such interventions may help to avert any long-term negative consequences for microbiota-gut-brain axis and behaviour.

3.4.6 STAR Methods

3.4.6.1 Animals

The experiments were performed in male NIH Swiss mice of different ages. Maternal care was a key consideration in our choice of strain, and the NIH Swiss outbred female mice are attentive mothers with a lower pup retrieval latency compared to B6 and 129Sv mice (Champagne et al., 2007). 8-week-old female and male breeders were obtained from Harlan laboratories, Oxford, UK. Breeding began after 1-2 weeks of acclimatization to the animal holding room. The animals were kept under a strict 12:12-h dark-light cycle, controlled temperature and humidity (20 ± 1 °C, 55.5%), with food and water given *ad libitum* unless specified. Male offspring were weaned on P21 and group-housed with 3-4 mice per cage. Experimental groups consisted of offspring from 3-10 litters (litter numbers for each experiment are specified in the figure legends). 10-week old Swiss mice, purchased from Harlan laboratories, UK, were used as conspecifics in the three-chamber sociability test. All procedures used in the present study were conducted in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposes and were approved by the Animal Experimentation Ethics Committee of University College Cork # 2012/036.

3.4.6.2 C-section surgery

Mice were time-mated, and the presence of a vaginal plug was marked as gestational day 0.5 (G0.5). Males were removed from the cage, and pregnant females were not disturbed unless for cage cleaning. At full term (G19.5) female mice were euthanized by cervical dislocation. To reduce bacterial contamination of the abdominal cavity, the abdominal skin was prepped by application of 70 % ethanol. The abdomen was incised, the uterus was removed and placed on sterile gauze. After this step, an incision was made in the uterus. To prevent hypothermia of the foetuses in the uterus, a heating pad was placed underneath to provide thermal support. The pups were then removed by gentle pressure with a sterile swab, and the umbilical cord was cut. Sterile cotton swabs were used to tear the amniotic membrane and massage each pup until spontaneous breathing was noted. The pups were given to a foster dam that gave birth on the same day. The pups were dried by smearing them with the bedding material from the cage of foster dam (CS). In addition, pregnant females were allowed to deliver spontaneously, and these litters were used as full-term vaginal delivery controls (VB). To control for the effects of fostering, a cross-fostered vaginally born group was included in the experimental design (CF, see next section) (Figure 1A).

3.4.6.3 Cross-fostering

Cross-fostering was performed on litters born within 12 h of each other. On the day of the birth, the litters were removed and put with their foster mothers. The pups were nursed by their respective foster mothers until weaning. Given that CF and VB animals showed a very similar behavioural phenotype across the lifespan, we focused on the VB control group for later experiments to meet the 3R requirements and minimise animal usage.

3.4.6.4 16S rRNA gene sequence-based microbiota analysis

Total DNA extraction from caecal and faecal matter was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Manchester, UK) coupled with an initial bead-

beating step. Extracted DNA was kept frozen at -20°C until further analysis. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol (http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). PCR was performed using forward primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and reverse primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Each 25 µL PCR reaction contained 5 ng/µL microbial genomic DNA, 1 µM of each primer and 12.5 µL 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd, Sigma, Dublin, Ireland.). The PCR conditions were as follows: initial denaturation at 95 °C for 3 min; 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Indianapolis, IN, USA). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, USA). Each 50 µL PCR reaction contained 5 µL purified DNA, 5 µL index primer 1 (N7xx), 5 µL index primer 2 (S5xx), 25 µL 2x Kapa HiFi Hot Start Ready mix and 10 µL PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Dublin, Ireland). 2 × 300 (bp) paired-end sequencing was performed on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

3.4.6.5 Microbiota bioinformatic sequence analysis

Paired-end sequences were assembled using FLASH (min overlap of 30 bp and min length of 460 bp) and analyzed using QIIME v1.8.0. Sequences were quality-checked and clustered into OTUs using USEARCH (v7.0-64bit). Taxonomic ranks were assigned

with a BLAST search against the SILVA SSU database v123. Alpha diversity indices were generated in QIIME.

3.4.6.6 Microbiota statistical analysis

Statistical analysis was done in SPSS (IBM, SPSS Statistics 24) and R software environment. The OTUs detected only in \leq two animals in each group were excluded from the analysis, as were the OTUs that did not give any BLAST hits or were unidentified or unknown on the genus level. Relative abundance of bacterial taxa on the phylum, family and genus level was expressed as % of identified sequences. Among-group differences in alpha-diversity indices and in the relative abundance of bacterial taxa were analysed with independent Mann-Whitney U test. p value < 0.05 was deemed significant; Benjamini-Hochberg (BH) adjustment with $Q=0.2$ was used to correct p values for multiple testing. p values are presented in **Tables S1 and S4**. For beta diversity, the Aitchison distance was calculated using the *ALDEx2* library in R to account for zeroes. Recommended settings were used, with 1000 permutations per sample. Variance-based Principal Component Analysis was done using the *prcomp()* function in R (version 3.5.1) using Rstudio (version 1.1.456). The *vegan* implementation of PERMANOVA followed by PERMANOVA as a post-hoc was used to test for differences on a beta-diversity level. Canonical correspondence analysis was performed using the *vegan* package in R (version 3.5). CCA plots on the OTU level were generated with the *vegan* library, ellipses represent 95% confidence interval visualised and calculated by the *ggplot2* library (ter Braak, 1986). In order to investigate the impact of co-housing on the microbiome in CS mice, a linear model was constructed based on the effect sizes for all identified bacteria in these groups in base R. Bacteria with the highest Cook's distance from the model were selected for further analysis. Samples with <40000 reads were excluded from the analysis (technical outliers).

3.4.6.7 Behavioural testing

The short and long-term effects of C-section on behaviour were evaluated in male offspring in early-life (P9/P10) and in adulthood (weeks 8-16). Mice were habituated to the behavioural room for 30 minutes prior to each test. The experimental procedures are described below; the experimental timelines are illustrated in **Figure 2A**, **Figure 5A** and **Figure 6B**. The order of behavioural tests and between-test recovery intervals were chosen to minimize the potential confounding carryover effects from the previous behavioural test. Behavioural tests were analysed by three independent experimenters blinded to experimental groups. All tests were performed during the lights on phase and between the hours of 9am and 2pm.

3.4.6.7.1 Isolation-induced ultrasonic vocalizations test

Isolation-induced ultrasonic vocalizations (USV) are produced by mouse pups during the first two weeks of life when separated from their mother and littermates (Winslow et al., 2000). USV was performed as described by Robertson et al. (Robertson et al., 2017). Pups were isolated and placed into a clean plastic container enclosed in a sound-attenuating chamber. Emission of USV calls were monitored by an ultrasound sensitive microphone – a bat detector (US Mini-2 bat detector, Summit, Birmingham, USA) tuned in the range of 60-80 kHz – suspended above the isolated pup for 3 min. The number of calls was recorded.

3.4.6.7.2 Maternal attachment test (homing test)

Maternal attachment test evaluates the ability of pups to differentiate their mother's and littermates' nest (Macri et al., 2010). Maternal attachment was evaluated accordingly to Morais *et al.* (Morais et al., 2018). At P10, the floor of a clean mouse cage was subdivided into three areas by wire-mesh dividers. One area was uniformly covered with home cage bedding, thus containing familiar odour stimuli. The opposite area was covered with bedding from the cage of another litter (born at approximately the same time). The middle section was covered with clean bedding material. Pups were placed individually in the middle section for 1 min; the dividers

were then removed, and the pups were allowed to freely explore the arena for 2 min. Total time spent in each area was recorded.

3.4.6.7.3 Three-chamber test

Sociability and social novelty recognition were evaluated as previously described by Desbonnet *et al.* (Desbonnet et al., 2014). Animals were placed in a rectangular apparatus divided into three chambers (left and right, and a smaller center chamber) with transparent partitions; small circular openings allowed easy access to all compartments. The test was composed of three sequential 10 min trials: (1) habituation (the test animal was allowed to explore three empty chambers); (2) sociability (an unfamiliar con-specific animal was placed in an inner mesh wire cage in either left or right chamber, the alternative chamber had an empty inner cage); (3) social novelty recognition (a novel con-specific animal was placed into the previously empty inner cage). All animals were age- and sex-matched; each chamber was cleaned and lined with fresh bedding between trials. For each of the three stages, behaviours were recorded by a video camera mounted above the apparatus; time spent in active exploration of inner cages (t) was measured and sociability index was calculated by using the formula $(t \text{ novel mouse} - t \text{ familiar mouse}) / (t \text{ novel mouse} + t \text{ familiar mouse})$.

3.4.6.7.4 Marble burying test

Marble burying test was used to measure anxiety-like behaviour, indicating higher levels of anxiety at higher number of marbles buried as described by Burokas et al. (Burokas et al., 2017). Clean cages were filled with a 4-cm layer of chipped wood bedding. Twenty glass marbles (15 mm diameter) were gently laid on top of the bedding, equidistant from each other in a 4×5 matrix arrangement. Each mouse was placed in the cage and allowed to explore it for 30 min. The number of buried marbles (> 2/3 marble covered by bedding material) was recorded.

3.4.6.7.5 Aversive open-field (OF) test

The aversive open-field (OF) test is used to assess the locomotor activity and the response to a novel stressful environment. Light was set at 1000 lux. A test mouse was placed in the center of an aversive open-field arena (Perspex box with white base: 30 x 30 x 20 cm) and was allowed to explore the arena for 10 min. The distance moved and time in zone were recorded using Ethovision videotracking system (Noldus Information Technology, Nottingham, UK). Using this technology, a center zone was demarcated as (20 x 15 cm, L x W). The time spent in each zone and the frequency of entries into each zone were also measured. The box was cleaned with 10% ethanol and allowed to dry between animals.

3.4.6.7.6 Novel object recognition test

Novel object recognition test was performed as Burokas et al. (Burokas et al., 2017). On day one, a test mouse was habituated to a square open-field box (Perspex sides and base: 34.5cm x 42.7 cm for mice) for 10 min in a dimly lit room (60 lux). On day two, two identical objects were positioned in adjacent corners of the arena approximately 5 cm from the walls, and the test mouse was placed in the arena for a 10 min exploration period. Following a 24 h inter-trial interval, one familiar object was replaced with a novel object, and the test mouse was introduced for a 10 min exploration period. Object exploration was defined as when the animal's nose comes within a 2 cm distance to the object. In-between trials, objects and testing arena were cleaned with 10% ethanol. Novel object recognition index was calculated by using the formula $(t \text{ novel object} - t \text{ familiar object}) / (t \text{ novel object} + t \text{ familiar object})$.

3.4.6.8 Statistical analysis for animal behavioural data

Statistical analysis was done in SPSS (IBM, SPSS Statistics 24) and R software environment (version 3.5.1). The normality of data distribution was checked with Shapiro-Wilk test, and the homogeneity of variances across groups was compared using Levene's test. For parametric data, two-tailed un-paired Student's T-test, two-tailed paired Student's T-test, one-way ANOVA followed by Tukey post-hoc tests or

two-way ANOVA with Tukey post-hoc tests were used to compare means between groups where appropriate. Extreme outliers were excluded when values exceeded $2 \times \text{Standard Deviation}$ from the *mean*. Technical outliers were excluded when animals were unable to perform the test. All parametric data were expressed as mean \pm S.E.M. For nonparametric data, a Kruskal-Wallis test and Mann-Whitney U test were used to compare differences across groups. Non-parametric data were expressed as median and interquartile range. p value < 0.05 was deemed significant; F and p values are presented in the figure legends or **Supplemental tables**. Mixed-effects regression model was used to re-analyse adult behavioural data using litter or cage as a fixed effect in order to examine the covariance structure that is inherent in the experimental design using R (version 3.4.1). p value < 0.05 was deemed significant; F and p values are presented in the **Table S3**.

3.4.6.9 Co-housing procedure

At three weeks of age, male offspring born by VB or CS were weaned, and mice were split across three different housing conditions: 1) VB group, where each cage consisted of three to four VB mice housed together (from 9 litters); 2) CS group, where each cage consisted of four CS mice housed together (from 6 different litters); 3) co-housing groups, where in each cage one CS born mouse was housed together with three VB mice (CS co-housed, from 7 litters and VB co-housed, from 10 litters). For this experiment, mice from different litters were randomly distributed across different cages and housing regimens. The co-housing system was adopted from Buffington et al. (2016) (Buffington et al., 2016). In the VB co-housed group, one animal per cage was randomly selected to pass through behavioural tests.

3.4.6.10 Quantitative determination of *Bifidobacterium breve* in faecal pellets

Absolute quantification of *Bifidobacterium breve* species was determined by quantitative PCR using genus-specific primers *Bifidobacterium spp.* F (5'-CTCCTGGAACGGGTGG-3') and R (5'-GGTGTCTCTCCCGATATCTACA-3'), (Matsuki et

al., 2002). Standard curves were created using bacterial DNA extracted from a pure culture of *Bifidobacterium longum* NCBIM8809 as previously reported (Arbolea et al., 2012) .

3.4.6.11 Probiotic and prebiotic administration

CS offspring were exposed to either probiotic *Bifidobacterium breve* M16V (*B. breve*), a commercially available probiotic and supplied by Danone Nutricia Research (Utrecht, The Netherlands) or a prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) (ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) starting from birth and throughout the experiment (**Table S5**). *B. breve* was given in drinking water at a concentration of 10^9 c.f.u./mL (freeze-dried bacterial stocks were re-suspended in MediDrop clear H₂O (75-02-1001), and drinking bottles were changed daily in the evening). Prebiotic mixture was given in the custom-made AIN93G rodent diet in a 9 (scGOS): 1 (lcFOS) ratio at the final concentration of 1%. Both interventions were given to the nursing dams starting from birth and throughout the lactation period till weaning. Male offspring were weaned on P21 onto the corresponding treatment. Control VB dams and offspring were given MediDrop clear H₂O as drinking water and AIN93G diet *ad libitum*.

3.4.6.12 Hippocampal RNA sequencing

Ventral hippocampus was dissected from adult VB, CF and CS mice (**Figure 2A**) using micro punch technique. Briefly, whole brains were snap-frozen on dry ice and stored at -80°C until ready for use. Using a mouse brain slicer , 1-mm thick sections were obtained from the entire brain, and micro punches of ventral hippocampal tissue were taken using a mouse brain atlas reference (Paxinos and Franklin, 2012). Total RNA was isolated from the ventral hippocampus using the mirVana™ miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific, Dublin, Ireland). RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®). RNA from hippocampal micro-punches from each group VB (N=5), CF (N=5) and CS

(N=6) were subsequently sequenced. Library preparation and sequencing, as well as Fastq-file generation was done by Beckman Coulter Genomics service (Danvers, MA, USA). Paired-end reads of 2×100 bp were produced on an Illumina HiSeq2500 sequencer.

3.4.6.13 Bioinformatic analysis pipeline

Fastq-format reads were quality filtered and trimmed using Trimmomatic (v0.32) with the following non-default parameters: *AVGQUAL*: 20; *SLIDINGWINDOW*: 4:20; *LEADING*: 10; *TRAILING*: 10; *MINLEN*: 60. Alignment to the mouse [reference genome](#) (GRCm38.p3) was achieved using the STAR aligner (v2.4.0f1) with default options and an index compiled with gene models retrieved from the Ensembl database (release 78). These gene models were also used for read counting for each gene using HTSeq-Count (v0.6.0) with the following non-default parameters: -s: no; -r: pos; -q -f bam -m intersection-nonempty. Differential gene expression was determined using the DESeq2 R-package (v1.6.2) with default parameters on pairwise comparisons of all possible group combinations. An adjusted p-value ≤ 0.1 (Benjamini-Hochberg method) was considered significantly differentially regulated. Raw and processed original data will be deposited in NCBI's Gene Expression Omnibus and made accessible through a unique GEO accession number upon publication.

3.4.6.14 Short chain fatty acid analysis

Caecal content (30-40mg) from adolescent and adult mice was vortex-mixed with 1ml Milli-Q water and incubated at room temperature for 10 min and subsequently centrifuged at 10,000g for 5mins to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear gas chromatography (GC) vial and 2-ethylbutyric acid (Sigma-Aldrich, Ireland) was added as an internal standard. Standard solutions of 10.0 m mol/L, 8.0m mol/L, 6.0m mol/L, 4.0m mol/L, 1.0m mol/L and 0.5m mol/L of acetic acid, propionic acid, isobutyric acid and butyric acid (Sigma-Aldrich), respectively were used for calibration. The concentrations of SCFA were measured using a Varian 3800 GC-flame-ionization system fitted with a ZB-FFAP column (30 m

X 0.32 mm X 0.25 μ m; Phenomenex, Macclesfield, Cheshire, UK). Initial oven temperature was set at 100°C for 30 secs and raised to 180°C at 8°C per min and subsequently held for 1 min, then increased to 200°C at 20°C per min and finally held at 200°C for 5 min. Helium was used as the carrier gas at a flow rate of 1.3ml/min. The temperature of injector and the detector were set at 240°C and 250°C respectively. A standard curve was constructed with different concentrations of a standard mix containing acetic acid, propionic acid, isobutyric acid and N-butyric acid (Sigma-Aldrich). Peaks were integrated using the Varian Star Chromatography Workstation v6.0 software (**Table S2**).

Chapter 4

4.1 Natural compulsive-like behaviour in the deer mouse (*Peromyscus maniculatus bairdii*) is associated with altered gut microbiota composition

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4.1.1 Abstract

Obsessive-compulsive disorder (OCD) is a psychiatric illness that significantly impacts affected patients, while available treatments yield suboptimal therapeutic response. Recently, the role of the gut-brain axis (GBA) in psychiatric illness has emerged as a potential target for therapeutic intervention. However, studies concerning the role of the GBA in OCD are limited. To investigate whether a naturally occurring obsessive-compulsive-like phenotype in a rodent model, i.e. large nest-building in deer mice, is associated with perturbations in the gut microbiome, we investigated and characterized the gut microbiota in specific pathogen free bred and housed large (LNB) and normal (NNB) nest building deer mice of both sexes ($n = 11$ per group, including 3 males and 8 females). Following baseline characterisation of nest building behaviour, a single faecal sample was collected from each animal and the gut microbiota analysed. Our results reveal the overall microbial composition of LNB animals to be distinctly different compared to controls (PERMANOVA $p < 0.05$). While no genera were found to be significantly altered after correcting for multiple comparisons, the normal phenotype showed a higher loading of *Prevotella* and *Anaeroplasma*, while the OC-phenotype demonstrated a higher loading of *Desulfovermiculus*, *Aestuariaispira*, *Peptococcus* and *Holdemanella* (cut-off threshold for loading at 0.2 in either the first or second component of the PCA). These findings not only provide proof-of-concept for continued investigation of the GBA in OCD, but also highlight a potential underlying etiological association between alterations in the gut microbiota and the natural development of obsessive-compulsive-like behaviours.

4.1.2 Introduction

Obsessive compulsive disorder (OCD) is a multidimensional psychiatric disorder that is characterised by intrusive and persistent thoughts or ideas, i.e. obsessions and/or ritualistic behaviours (compulsions) that are often expressed in an attempt to mitigate the level of distress caused by the obsessive thoughts (Apa, 2013; Abramowitz and Jacoby, 2015; Wu and Lewin, 2017). The condition is phenotypically

heterogeneous and symptoms usually cluster along the basis of specific themes, e.g. fears of contamination and cleaning rituals, fears of harm and checking compulsions, a need for symmetry and just-right feelings associated with ordering compulsions, as well as covert inappropriate thoughts relating to, among others sexual misconduct, religion, and violence (Williams et al., 2013; Abramovitch and Cooperman, 2015).

Chronic high-dose selective serotonin reuptake inhibitors (SSRIs) are currently recommended as first line pharmacotherapy for OCD (Albert et al., 2018), while increasing the dose of the SSRI used or SSRI-antipsychotic augmentation strategies are clinically employed in the treatment of SSRI-refractory OCD (Dold et al., 2015). Nevertheless, a significant number of patients respond poorly to these options and other available pharmacotherapeutic interventions (Albert et al., 2018). Therefore, a better understanding of the etiology and pathophysiological processes underlying OCD is needed to develop more effective treatment options.

During the past decade, the gut-brain axis (GBA) and its involvement in psychiatric disease has gained significant interest (Mayer et al., 2014; Dinan and Cryan, 2017; Bastiaanssen et al., 2018). Communication between the gut and the brain takes place on a number of functional levels, including neural, neuroendocrine and immunological signalling (Foster and Neufeld, 2013; Cusotto et al., 2018). Indeed, the gut microbiota is regarded as one of the major immunomodulatory influences in the human body (Zhao and Elson, 2018). While the exact etiological associations between psychiatric illness and the gut microbiota are still not fully understood, altered immune responses may be instrumental in the pathogenesis of brain disorders, including OCD (da Rocha et al., 2008; Turna et al., 2016). In light of this, several investigations into microbiota manipulation in animal models of psychiatric illness have been conducted, including models of depression (Kelly et al., 2016), anxiety (Crume yrolle-Arias et al., 2014), and OCD (Kantak et al., 2014). However, the exact translational value of these findings remains to be established (Kelly et al., 2016).

Regarded collectively, natural compulsive-like behavioural phenotypes expressed by deer mice (*Peromyscus maniculatus bairdii*), i.e. large nest building (LNB) and high stereotypy, provide a well-validated naturalistic pre-clinical framework in which to

study the etiopathology of OCD (Scheepers et al., 2018). Approximately 30% of laboratory housed deer mice of both sexes express LNB (Wolmarans et al., 2016). LNB manifests by the age of 8 weeks and is persistent and repetitive over the course of several trials. From a teleonomic perspective (Thornhill, 1996), LNB can be regarded as a maladaptation in a specific component of the normal behavioural repertoire of deer mice. Indeed, considering that mice build nests for the purposes of safety and protection, temperature regulation and to provide adequate nurseries for offspring (Jirkof, 2014), excessively large nests expressed in the laboratory serve no unique purpose. From an evolutionary perspective, nesting size and quality play a major role in mate choice and reproductive success in some species, e.g. birds (Holbeck and Riebel, 2009), fish (Jamieson, 1995), and frogs (Felton et al., 2006). However, this is not true for mice, which generally exercise mate choice by random or on the basis of amongst others, dominance, probability of genetic success, overall health, or patterns of ultrasonic vocalization (for a detailed review, see Latham and Mason (2004)). That both male and female deer mice engage in LNB, also excludes the likelihood of sex-related differences in nesting phenotype. Therefore, it is likely that excessive nest-building is expressed at the cost of other functions for which effort, time and energy is required, and may thus be regarded as a naturalistic maladaptation (Crespi, 2000).

We have previously shown that LNB is responsive to chronic high-dose (50 mg/kg/day) oral treatment with the SSRI, escitalopram (Wolmarans et al., 2016). This is in line with the therapeutic, albeit varying, effect of SSRI intervention observed in the majority of patients with OCD (Fineberg et al., 2006). Moreover, normal nest building (NNB) and LNB can be separated based on the underlying involvement of serotonin in its expression, as NNB remains wholly unaltered following such intervention, with nests neither decreasing or increasing in size (Wolmarans et al., 2016). Such difference in treatment response also imply that it is not the behavioural act of nesting per se, but rather the aberrant cognitive processes that underlie such behaviour, that are modified by serotonergic intervention.

Considering the literature reviewed here and the fact that LNB spontaneously manifest in a sub-population of deer mice only, the present work aimed to

investigate whether an association between such behaviour and natural modifications in the gut microbiota may exist. In fact, bearing in mind that theories pertaining to the involvement of the gut-brain axis in psychiatric illness point to developmental relationships (Borre et al., 2014), the study of parallel, but equally intrinsic and non-induced changes in both behaviour and the gut microbiome may provide valuable insight into our understanding of neurodevelopmental psychiatric conditions.

4.1.3 Materials and Methods

4.1.3.1 Animals

Deer mice of both sexes were obtained from the deer mouse colony of the North-West University (NWU), Potchefstroom, South Africa (ethical approval number: NWU-00284-17-S5; AnimCare Research Ethics Committee, National Health Research Ethics Committees Registration Number: AREC-130913-015). The original breeding pairs were established using animals obtained from the *Peromyscus* Genetic Stock Centre at the University of South Carolina, USA. Since only 30% of deer mice express LNB behaviour (Wolmarans et al., 2016), 40 deer mice (age 12 weeks) were screened to identify 11 (3 male and 8 female) deer mice of each nesting cohort, i.e. NNB and LNB. In line with the minimum recommendations set by the ARRIVE-guidelines (Kilkenny et al., 2010), and considering an extensive review of recent literature (Cani et al., 2008; Chang et al., 2015; Desbonnet et al., 2015; Luczynski et al., 2016; Sampson et al., 2016), group sizes of 3 – 8 animals are deemed sufficiently powerful in pre-clinical microbiome investigations.

Animals were bred according to a standard out-bred protocol (Bateson, 1983) and housed and maintained in the specific-pathogen-free (SPF) area of the Vivarium at the North-West University, Potchefstroom, South Africa. The initial breeding pairs—used to breed the 40 mice screened in this investigation—were randomly allocated without prior knowledge of their nest building profiles. After weaning, offspring were housed in same-sex groups (4 – 6 animals per cage) in individually ventilated cages [(35cm (l) x 20cm (w) x 13cm (h); Techniplast® S.P.A., Varese, Italy)], until one week prior to the onset of the first nest building analysis. From this point onwards,

each animal was allocated to its own cage, while all experimental analyses were conducted in these same cages throughout the investigation. Animals were kept on a 12-hour light/dark cycle (06h00/18h00), at a temperature of $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 5\%$. Food and water were provided ad lib. All mice received food from the same batch of pelleted rodent chow throughout the study. Cages were cleaned and new bedding material, consistently taken from the same batch of ground corncob, added once a week on the same day.

4.1.3.2 Nest building analysis

Nest building behaviour was quantified as described previously (Wolmarans et al., 2016). In short, nesting behaviour was assessed in each animal for 7 consecutive 24-hour periods. An excess of pre-weighed, sterile, hospital-grade, non-scented cotton wool was introduced in the roof of each home cage every day between 15h00 and 16h00. As mice generally build their nests just before dawn (Jirkof, 2014), the remaining cotton wool was only removed and weighed between 13h00 and 14h00 on the following day. Each day, built nests were removed, discarded and additional pre-weighed cotton wool supplied. Animals did not have access to any other form of nesting material, and food and water were supplied as normal. Daily nesting scores were expressed in grams of cotton wool used with a cumulative nesting score, describing nesting size and not quality per se, determined after one week (Wolmarans et al., 2016). As nest building is a natural behaviour expressed by all rodents (Smithers, 1983), only animals that consistently built large nests over the course of 7 days were included in the LNB cohort. This was determined by plotting the total nesting scores against the coefficients of variance with respect to daily nesting behaviour, where LNB was defined as nesting behaviour that clustered within, or as near to, the upper quarter of the nesting score distribution, while demonstrating the lowest degree of variance (Figure 4.1-1). Likewise, NNB animals were identified as those individuals that built the smallest nests consistently over the course of 7 days—for a full review of this methodology, please refer to Wolmarans et al. (2016). A clear separation of the LNB and NNB cohorts (Figure 4.1-2i – 2iv) is important in order to directly relate the compulsive-like phenotype to perturbations in the gut microbiota. As such, a group of mice that not only built smaller nests than

the LNB group, but also did so with the lowest possible degree of variability (Figure 4.1-1), were set as control. That said, while not a single animal refrained from engaging in nesting behaviour (Figure 4.1-1), NNB animals expressed nest building behaviour in a more adaptable manner (as indicated by the higher degree of between-day variance compared to LNB animals; Figure 4.1-1). These characteristics in nesting phenotype point to a clear separation between the two cohorts, as LNB animals not only express the highest nesting scores but do so without significant between-day variation. In other words, the motivational drive to engage in LNB is not only observed during a few nights of the 7-day assessment period, but across most of the trials (Figure 4.1-1), pointing to a OCD-like phenotype akin to behavioural inflexibility (Gillan et al., 2011).

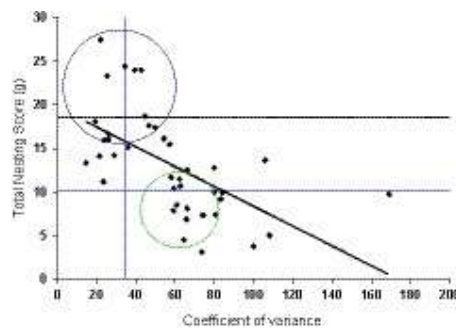


Fig. 4.1-1 Plot of the average total nesting scores (g) generated by each animal over the course of 7 days and the coefficients of variance with respect to the between-day nesting scores. Horizontal dotted lines intersecting the y-axis indicate the 25th and 75th percentiles of with respect to the total nesting scores generated. The vertical dotted line intersecting the x-axis indicates the 25th percentile with respect to the coefficients of variance. Blue circle: animals identified as LNB; Green oval: animals identified as NNB.

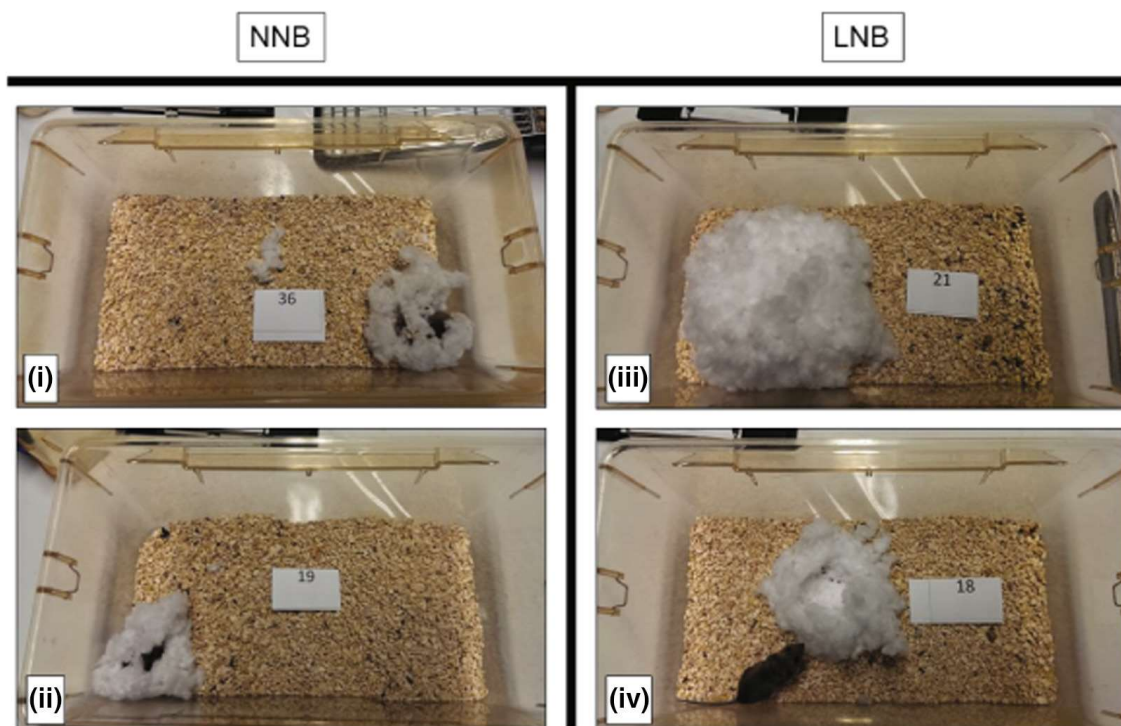


Fig. 4.1-2 Photographs of typical nests built by NNB (i, ii) and LNB (iii, iv) animals, respectively.

4.1.3.3 DNA analyses

4.1.3.3.1 Sample collection and DNA extraction

Fresh faecal samples were collected during the first hour of the dark (wake) cycle with a sterilized tweezer, transferred to 1.5ml Eppendorf® Safe-Lock tubes and immediately flash frozen in liquid nitrogen (Hong et al., 2010). Samples were kept frozen at -80°C until the extraction of DNA. The QIAamp® PowerFecal® DNA kit (QIAGEN, Valencia, CA, USA) was used to extract the microbial DNA from faecal samples (0.25g/sample). DNA extraction was performed as per the manufacturer's instructions to ensure maximal cell lysis of bacterial cell wall components. The Thermo-Scientific® NanoDrop One Microvolume UV-Vis Spectrophotometer was used to assess the quality and quantity of the extracted microbial DNA.

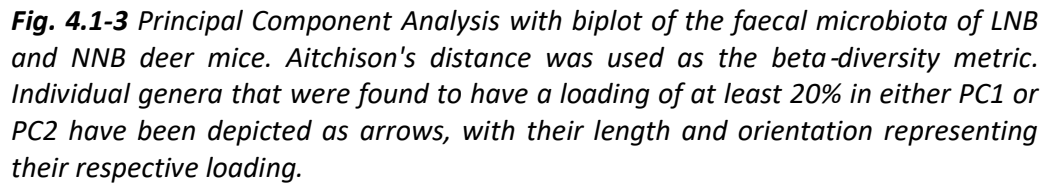
4.1.3.3.2 DNA sequencing

Paired-end sequencing of the V3 to V4 hypervariable regions of the 16S rRNA was performed by Macrogen® Inc. (South-Korea) using the FWD 5'-CCTACGGGNGGCWGCAG-3' and REV 5'-GACTACHVGGGTATCTAATCC-3' primer pair

on a MiSeq (Illumina) platform. The V3 to V4 hypervariable region was used as high inter-taxon variability of these regions can be used to distinguish between closely related bacteria. Library preparation was performed as per the 16S Metagenomic Sequencing Library, Preparation Part #15044223, Rev. B protocol, except when using the Hercules II Fusion DNA Polymerase (Agilent, Santa Clara, USA), the Nextera Index Kit V2 (Illumina, San Diego, USA) and the V3-V4 primers. Indexed adapter-ligated fragments were pooled and then gel purified and PCR amplified.

4.1.3.3.3 Statistical analysis

First, quality control of raw *fastq* sequencing files was performed using *fastqc* and *multiqc* programs respectively (Ewels et al., 2016). Second, a Divisive Amplicon Denoising Algorithm (DADA) 2 (version 1.8) (Callahan et al., 2016) in R studio (R version 3.4.3; R-studio version 1.1.456) (Gandrud, 2016) was used for the construction of an amplicon sequence variant (ASV) table (Callahan et al., 2016). The DADA 2 workflow consisted of the following steps: inspecting the read quality profiles, filtering and trimming low-quality reads, identifying error rates, dereplication (eliminating redundant comparisons), sample inference, merging paired reads, constructing an amplicon sequence variant (ASV) table, removing chimera's and assigning taxonomy. Taxonomy was assigned using the Ribosomal Database Project (RDP) as a reference database (Cole et al., 2005). The *vegan* package in R was used to evaluate β -diversities. For PCA, Aitchison distance was calculated using the *ALDEx2* library (Fernandes et al., 2014; Gloor et al., 2017). The Shannon, Simpson, Chao1, Observed and Fisher diversity indices were used to evaluate α -diversity. Filtering was performed to only include taxa that were observed more than once in at least 15% of the animals. Centred log-ratio transformed (clr) relative abundance for each ASV was also determined using *ALDEx2*. To test for statistically significant differences in the relative abundance of ASVs between the gut microbial composition of NNB and LNB animals, we used permutational multivariate analysis of variance (PERMANOVA; *vegan* package). The Mann-Whitney U test was used to compare alpha diversity metrics, a *p*-value of < 0.05 was deemed significant in all cases.



To test for differences between the gut microbial composition of NNB and LNB animals (3 male and 8 female animals per group, respectively), a total of 22 faecal samples were analysed, each from the same time point in the sleep cycle. All samples passed quality control (QC) with a minimum read count threshold of 10 000 and median read depth of 34 686 reads per sample. From this, 86 genera were detected.

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4.1.5 Discussion

The major findings of the present work are that 1) there is a significant difference in the overall gut microbiota composition of NNB and LNB animals, and 2) such difference is driven in NNB and LNB animals by the prevalence of *Prevotella* / *Anaeroplasma*, and *Desulfovermiculus* / *Aestuariispira* / *Peptococcus* / *Holdemanella*, respectively.

The neurobiological and pathophysiological processes underlying OCD are not yet fully elucidated, with current treatment options also yielding suboptimal results (Atmaca, 2016). During the past decade, our understanding of the GBA has expanded significantly (Sherwin et al., 2018), having been shown to play a role in the pathophysiology of a number of psychiatric illnesses, including anxiety and depression (Foster and Neufeld, 2013). In terms of OCD, very little research has been conducted to elucidate if and how the gut microbiota may be associated with the condition (Kantak et al., 2014; Turna et al., 2016). Further, although some clinical results have been reported that may be indicative of the potential therapeutic value of microbiotic modification in the treatment of central nervous system disorders (Messaoudi et al., 2011), it remains difficult to translate these findings to clinical studies (Kelly et al., 2016).

In this investigation we interrogated possible associations between a *naturally* developing compulsive-like phenotype, i.e. LNB in the deer mouse (Wolmarans et al., 2016), and alterations in the gut microbiota. Our finding that the community composition of the gut microbiota in LNB animals is significantly different from that in the NNB cohort is noteworthy. Taking into account that LNB transpires naturally over the course of development and given that animals included in this investigation have been randomly selected without litter bias and housed individually, the differences observed in microbial composition parallel the differences observed in behavioural expression; this association is therefore likely naturalistic. Our finding that a clustering of *Prevotella* and *Anaeroplasma* was driving the compositional ordination in NNB compared to LNB animals, is noteworthy. Interestingly, while the human gut microbiota demonstrates significant biogeographical stratification

(Donaldson et al., 2016), most organisms cluster within the phyla *Firmicutes* and *Bacteroidetes*, which includes the genus *Prevotella*, and (Albenberg and Wu, 2014; Marchesi et al., 2016). Further, both *Prevotella* (Shenker et al., 1991) and *Anaeroplasma* (Beller et al., 2019) have been associated with significant anti-inflammatory properties, while children diagnosed with autism, a condition also characterized by persistent behavioural phenotypes, have been shown to present with lower gut *Prevotella* abundance (Kang et al., 2013).

In terms of the gut microbiota composition of LNB animals, members of the phylum Proteobacteria, of which *Aestuariispira* is an example, have been proposed as microbial signatures of among others, inflammatory conditions (Rizzatti et al., 2017). Further, hydrogen sulphide releasing bacteria, of which *Desulfovermiculus* (Loubinoux et al., 2002), and *Peptococcus* (Bourgault and Rosenblatt, 1979; Van Eldere et al., 1988) are examples, have been associated with gut mucosal injury and inflammatory pathology (Loubinoux et al., 2002). Interestingly, *Peptococcus*, has also been implicated in other models of adult neurodevelopmental aberrancies following exposure to prenatal stress (Golubeva et al., 2015). Therefore, considering that LNB develops spontaneously over time, and that this phenotype is associated with a lower loading of *Prevotella* and *Anaeroplasma*, it is possible that the composition of the microbiota in LNB animals reported here, can exert a gut-to-brain neuroimmune-associated etiological influence on the expression of compulsive-like nest building in the deer mouse (Heijtz et al., 2011; Furtado and Katzman, 2015; Turna et al., 2016). This possibility should be elaborated in future investigation.

4.1.6 Conclusion

The data presented here indicates for the first time in a pre-clinical model that naturally developing OC-like behaviour is associated with inherent differences in the gut microbial composition, compared to that in normal controls. Future investigations into a possible causal role of the gut microbiota in the etiology of compulsive phenotypes are warranted. Specifically, the relationship between obsessive-compulsive behaviour, stress, and immune alterations on the one hand, and adaptations in the microbiota of normal and compulsive-like animals on the

other, needs further elucidation. Further, using gnotobiotic mice and other means of microbiota modification, e.g. antibiotic treatment, it would be of value to characterize the behavioural response in LNB deer mice under circumstances of microbiota alterations. Such studies will potentially contribute to a better understanding of the neurobiology underlying OCD and may ultimately lead to the development of better treatment.

Chapter 5

5.1 Volatility as a Concept to Understand the Impact of Stress on the Microbiome

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5.1.1 Abstract

The microbiome-gut-brain-axis is a complex phenomenon spanning several dynamic systems in the body which can be parsed at a molecular, cellular, physiological and ecological level. A growing body of evidence indicates that this axis is particularly sensitive to the effects of stress and that it may be relevant to stress resilience and susceptibility. Although stress-induced changes in the composition of the microbiome have been reported, the degree of compositional change over time, which we define as volatility, has not been the subject of in-depth scrutiny. Using a chronic psychosocial stress paradigm in male mice, we report that the volatility of the microbiome significantly correlated with several readouts of the stress response, including behaviour and corticosterone response. We then validated these findings in a second independent group of stressed mice. Additionally, we assessed the relationship between volatility and stress parameters in a cohort of health volunteers who were undergoing academic exams and report similar observations. Finally, we found inter-species similarities in the microbiome stress response on a functional level. Our research highlights the effects of stress on the dynamic microbiome and underscores the informative value of volatility as a parameter that should be considered in all future analyses of the microbiome.

5.1.2 Introduction

The mammalian gut plays host to approximately 1 trillion microbial organisms collectively known as the gut microbiome (Sommer and Bäckhed, 2013). The microbiome is highly sensitive and reactive to the effects of stress to the extent that it is now accepted that the stress response is not solely the domain of brain function, but rather that it results from a synergy of mechanisms that constitute the gut-brain axis (Dinan and Cryan, 2012; Foster et al., 2017; Bastiaanssen et al., 2018; Cryan et al., 2019; Bastiaanssen et al., 2020; Cruz-Pereira et al., 2020). In particular, studies in rodents have correlatively linked alterations in microbiota composition to the effects of stress on behaviour (Bharwani et al., 2016; Bharwani et al., 2017; Burokas et al., 2017; Marin et al., 2017; Szyszkowicz et al., 2017; Xu et al., 2020) and the central/peripheral inflammatory milieu (Bailey et al., 2011; Bharwani et al., 2016;

Bharwani et al., 2017; Burokas et al., 2017; Szyszkowicz et al., 2017). Going further, manipulation and perturbation of the microbiome have been shown to alter the reaction to stress, further solidifying the regulatory role of the microbiome in the stress response (Jašarević et al., 2017; Langgartner et al., 2018; Pearson-Leary et al., 2019; Provensi et al., 2019; Stothart et al., 2019; Donoso et al., 2020; Kuti et al., 2020; Morais et al., 2020; Wang et al., 2020). Moreover, in humans there have been a number of studies confirming a relationship between stress and microbiome composition across the lifespan (Messaoudi et al., 2011; Zijlmans et al., 2015; Allen et al., 2016; Hemmings et al., 2017; Papalini et al., 2019).

These observations notwithstanding, a common feature of the above cited studies is the fact that they are a ‘snapshot-in-time’ analyses of samples and therefore do not consider the compositional and temporal instability of the microbiome (Caporaso et al., 2011; Claesson et al., 2011). Temporal variance as a feature of the microbiome has been discussed before. Initially, the volatile nature of the microbiome was pointed out (Weinstock, 2011) and relatively soon thereafter, the term volatility was used to refer to the degree of change between timepoints (Goodrich et al., 2014). In the context of stress, recent studies have shown differential effects on microbiota beta-diversity dependent on when the samples were taken – shortly after commencement of social stress or towards the end of the protocol (Gautam et al., 2018), though the degree of change was not pursued, nor linked to the severity of the stress response. Recently, in a large human cohort, patients with Inflammatory Bowel Disease (IBD), a condition that is associated with an increased level of stress, were shown to have a more volatile microbiome than healthy volunteers (Clooney et al., 2020; Ryan et al., 2020). While these studies provide some evidence that volatility maybe related to stress, the impact of stress on microbial volatility measures remain unknown. Furthermore, deciphering what makes a microbial community volatile can provide new insights into its role in mediating the effects of stress. To further investigate volatility in relation to stress, we exposed mice to chronic psychosocial stress and assessed volatility. Based on the findings of this discovery cohort, we then set out to validate our findings in an independent cohort of a different strain of mice

under the same experimental conditions. Finally, we also took advantage of an ongoing study involving healthy volunteers undergoing academic examination stress and sought to examine whether the interaction between volatility and stress occurred across species.

5.1.3 Materials & Methods

5.1.3.1 Animals

For this study we used two cohorts of adult male mice. The first cohort will be referred to as the Discovery cohort and consisted of (B6;129-*Gt(ROSA)26Sor^{tm1(CAG-cas9,-EGFP)}Fezh*/J; <https://www.jax.org/strain/024857>). The second cohort will be referred to as the Validation cohort and consisted of adult male C57BL/6 (Envigo, UK). There were no differences in terms of experimental treatment and handling between the two cohorts. There were three weeks between the arrival of the animals and the start of singly housing. Approximately one week before commencement of social defeat sessions, all mice were singly housed and weighed daily over the course of the experimental protocol (Figure 5.1-1). For the chronic social defeat stress procedure, non-experimental singly housed adult male CD1 were used as aggressors (Envigo, UK). Mice were kept under a 12 hr light/dark cycle (ON 7:30AM, OFF 7:30PM) in a temperature/humidity controlled environment (21°C, 55.5%) with food and water *ad libitum*. The main behavioural and physiological responses to chronic stress of the Discovery cohort have been initially reported elsewhere (Gururajan et al., 2019) and are used here in a correlative capacity with the microbiome analysis.

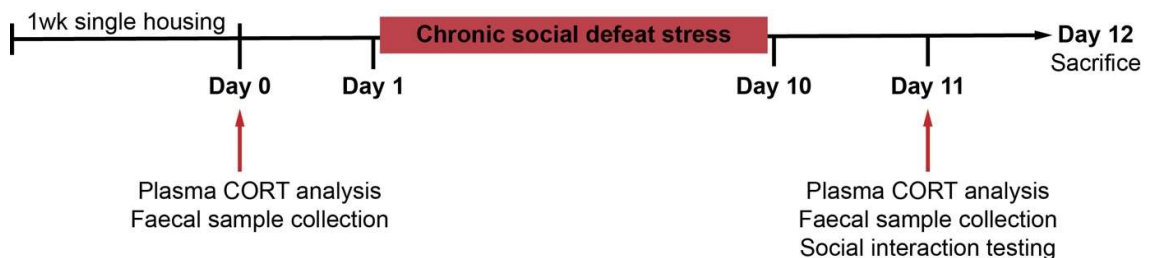


Figure 5.1-1: Mouse experimental timeline. Mice were singly housed for 1 week prior to the first stool and plasma collection on Day 0. From Day 1 to 10, mice were randomly assigned to either the control condition (not shown) or chronic social defeat stress. On Day 11, plasma and faecal boli samples were again collected and social behaviour was assessed. The following day, all mice were culled. Trunk blood was collected for flow cytometry and brain tissue was processed for gene expression analyses.

5.1.3.2 Chronic social defeat stress

Mice were randomly assigned to either the social defeat stress or control groups. Defeat sessions were performed as previously described (Gururajan et al., 2019). Control mice remained in their home-cages over the course of the stress protocol but were handled to an equal extent as the stressed mice in the process of measuring daily body weight and collecting tail-blood samples. Across the duration of the defeat protocol, to prevent contamination during defeat procedures, the experimenter removed any traces of faecal boli produced by the aggressor or the stressed mice. Based on the findings in the first cohort of mice, we repeated the experiment in a larger cohort. We refer to these cohorts as the Discovery cohort and the Validation cohort, respectively. Further details can be found in Supplementary Methods.

5.1.3.3 Social interaction test

The social interaction testing of mice was used to assess avoidance of the CD1 aggressors the day after the last defeat session and was carried out as previously described (Gururajan et al., 2019). Social interaction (SIT) ratios were generated based on social investigation time in an arbitrarily defined interaction zone. Further details can be found in Supplementary Methods.

5.1.3.4 Plasma sampling for corticosterone

Collection and analysis of plasma samples for corticosterone was carried out as previously described (van de Wouw et al., 2018; Gururajan et al., 2019). Briefly, tail bleeds were carried out within 1 hour of the lights turning off (1930-2030). Whole blood was collected in sterile eppendorfs. Whole blood was centrifuged (3500g, 10 minutes, 4 degrees C) and plasma was collected. Plasma samples were analyzed in duplicate using the Enzo® Corticosterone ELISA kit plate (ADI-900-097, Enzo, Exeter, United Kingdom) according to the manufacturer's instructions. ELISA plates were read using a Multiskan® microplate photometer (Thermofisher Scientific®, Waltham, MA, USA) at 405 nm. See Supplementary Methods for further detail.

5.1.3.5 Human Study

Briefly, healthy volunteer study participants were recruited via advertisement and direct contact to the student population of University College Cork (UCC). A total of 84 volunteers responded to advertisement and direct contact; 54 were pre-screened by telephone call (64%); 36 were invited to a screening visit (43%); and thirty were enrolled in the study and randomised to treatment (36%). Inclusion criteria: participant must be able give written informed consent; be between 18 and 30 years of age; be male; be in generally good health as determined by the investigator. Prior to testing days, participants were asked to refrain from strenuous exercise and alcohol 24 hours before the session, and from caffeine three hours prior to the session. At the screening visit, two weeks before baseline measurement, study participants were asked about their demographics, general medical history, medication record, and other metadata. Furthermore, the participants were screened using the MINI International Psychiatric Interview (to exclude subjects with a significant DSM-V psychiatric diagnosis). Participants attended for study visits during 2 semesters in UCC – both 8 weeks prior to an exam period and during an exam period. The exam visit took place during the participant's exams, but not on the day of an exam. The measures taken during the visit included Cohen's Perceived Stress Scale (PSS). Faecal samples from the morning of the visit were collected into plastic containers containing an Anaerogen sachet. Participants were instructed to keep the sample in a cool place until delivery at the study visit. Salivary Cortisol Awakening Response was assessed using the Cortisol ELISA kit plate ADI-900-071, Enzo, Exeter, United Kingdom) according to the manufacturer's instructions. ELISA plates were read using a Multiskan® microplate photometer (ThermoFisher Scientific®, Waltham, MA, USA) at 405 nm.

5.1.3.6 16S rRNA Gene Sequencing

DNA was extracted from faecal samples and prepared for sequencing using an Illumina 16S Metagenomic Sequencing Library Protocol. See supplementary methods for further details.

5.1.3.7 Bioinformatics analysis

Three hundred base pair paired-end reads were pre-filtered based on a quality score threshold of >28 and trimmed, filtered for quality and chimaeras using the DADA2 library in R (version 3.6.3). Only samples with >10.000 reads after QC were used in analysis. Taxonomy was assigned with DADA2 against the SILVA SSURef database release v138. Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. ASVs were aggregated at genus level; those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as non-zero in 10% or few of total samples.

5.1.3.8 Statistical analysis

Further data-handling was done in R (version 3.6.3) with the Rstudio GUI (version 1.1.453). Custom R scripts are available at <https://github.com/thomazbastiaanssen/Tjazi> (Bastiaanssen, 2018). Stacked barplots were generated by normalizing counts to 1, generation proportions. Genera that were never detected at a 1% relative abundance or higher were aggregated and defined as Rare taxa for the purposes of the stacked barplots. Principal component analysis was performed on centred-log ratio transformed (clr) values using the ALDEx2 library (Fernandes et al., 2014). Number of permutations was always set to 1000. Volatility was measured as distance between before and after the experiment or treatment and was calculated as the Aitchison distance between the two timepoints. Unlike other distance metrics such as Bray-Curtis and Jensen-Shannon divergence, the Aitchison distance takes into account the compositional nature of microbiome datasets (Aitchison et al., 2000). Piphillin was used for functional inference from 16S rRNA gene sequence of mouse stool samples in the form of KEGG orthologues (Iwai et al., 2016). Gut-Brain Modules (GBMs) and Gut-Metabolic Modules (GMMs) were calculated using the R version of the Gomixer tool (Valles-Colomer et al., 2019). Differential abundance of both microbes and functional modules were calculated using implementations of the ALDEx2 library. As part of testing for correlations between volatility and metadata, skadi, an implementation of jackknifing and Grubb's test, was used to assess reliability of the data and detect

outliers (R scripts available). Correlation was assessed using Spearman's rank correlation coefficient in the case of low N or heteroskedacity. In all other cases the Pearson correlation coefficient was used. Normality was assessed using the Shapiro-Wilk test. For normally distributed data, between-group differences were analysed using ANOVA or unpaired two-tailed t-test and Tukey's test for post-hoc analysis. For datasets in which the condition of normality was violated the non-parametric Kruskal-Wallis test was used and post-hoc analysis was done using the Wilcoxon test. A p-value of <0.05 was deemed significant in all cases. To correct for multiple testing in tests involving microbiota or Functional Modules, the Benjamini-Hochberg (BH) post-hoc was performed with a q-value of 0.1 as a cut-off.

5.1.4 Results

5.1.4.1 The gut microbiome is differentially volatile in response to chronic social defeat stress.

To quantify volatility, which we define as the degree of compositional change of the microbial ecosystem over time, we calculated the intra-subject Aitchison distance between the genus-level count tables from the same subject taken before and after the experiment using the clr-transformation (Figure 5.1-2C). Stressed mice showed a trend toward a significantly higher degree of volatility when compared to controls in the Discovery cohort (Figure 5.1-2D). In the validation cohort, we again found a higher degree of volatility in stressed animals compared to controls, this time significantly so (Figure 5.1-2E-F).

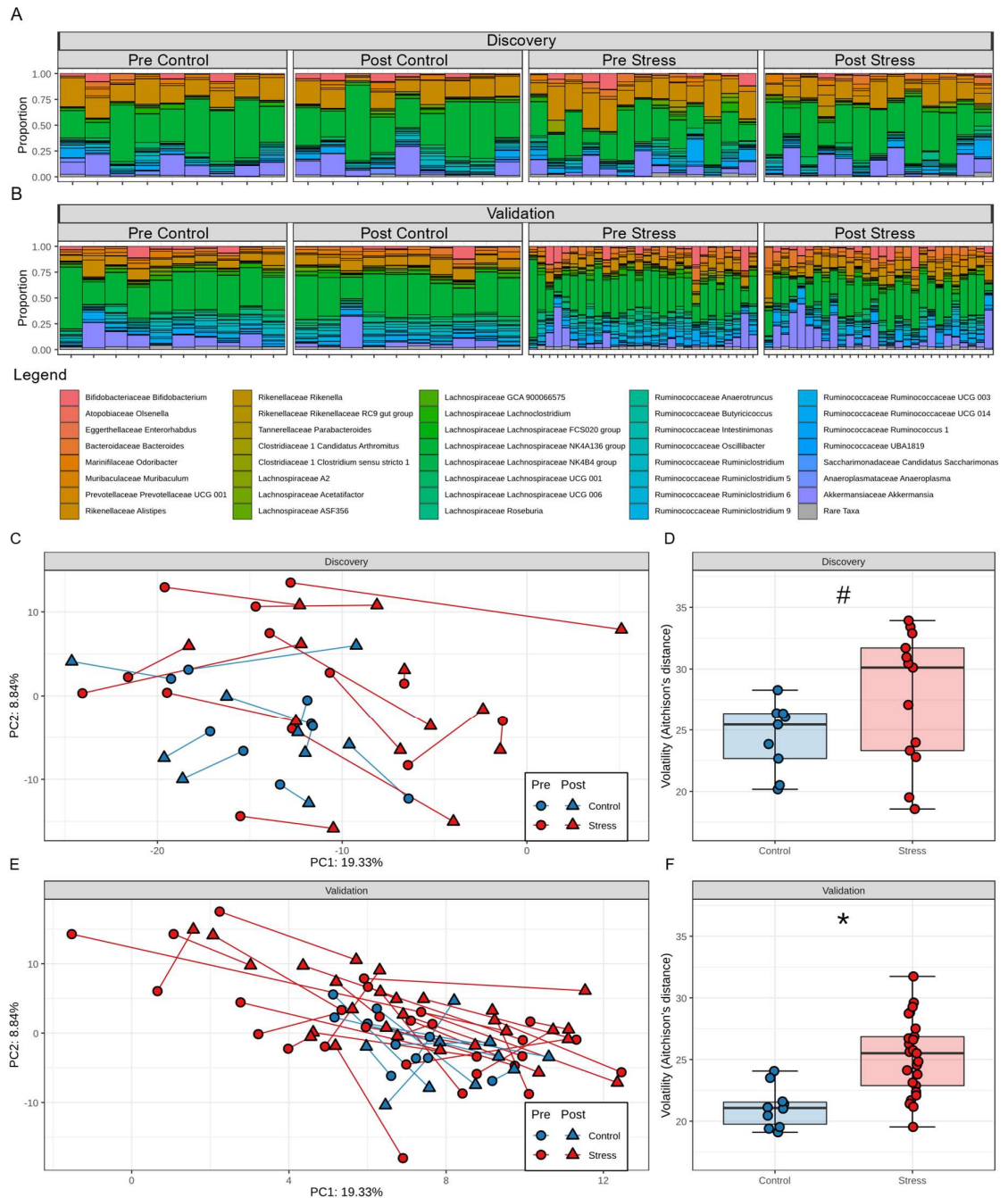


Figure 5.1-2: Microbial volatility is influenced by stress. (A) Stacked barplot showing the proportion of genera based on 16S sequences detected per sample in the Validation cohort and the (B) Discovery cohort. Volatility was defined as the Aitchison distance travelled over the 10-day experiment. (C) PCA showing the microbiome compositions of animals before and after the 10-day period. Lines link the same animal over time, showing the trajectory and distance travelled in time. (D) Aitchison distance travelled is shown on the y-axis; Mann-Whitney $p=0.093$. (E) The PCA of the validation cohort and (F) corresponding elevated volatility in stressed mice.; Mann-

Whitney $p=5.06 \times 10^{-5}$. Discovery cohort: Control $N = 9$; Stress: $N = 13$, validation Control cohort: $N = 10$; Stress: $N = 28$.

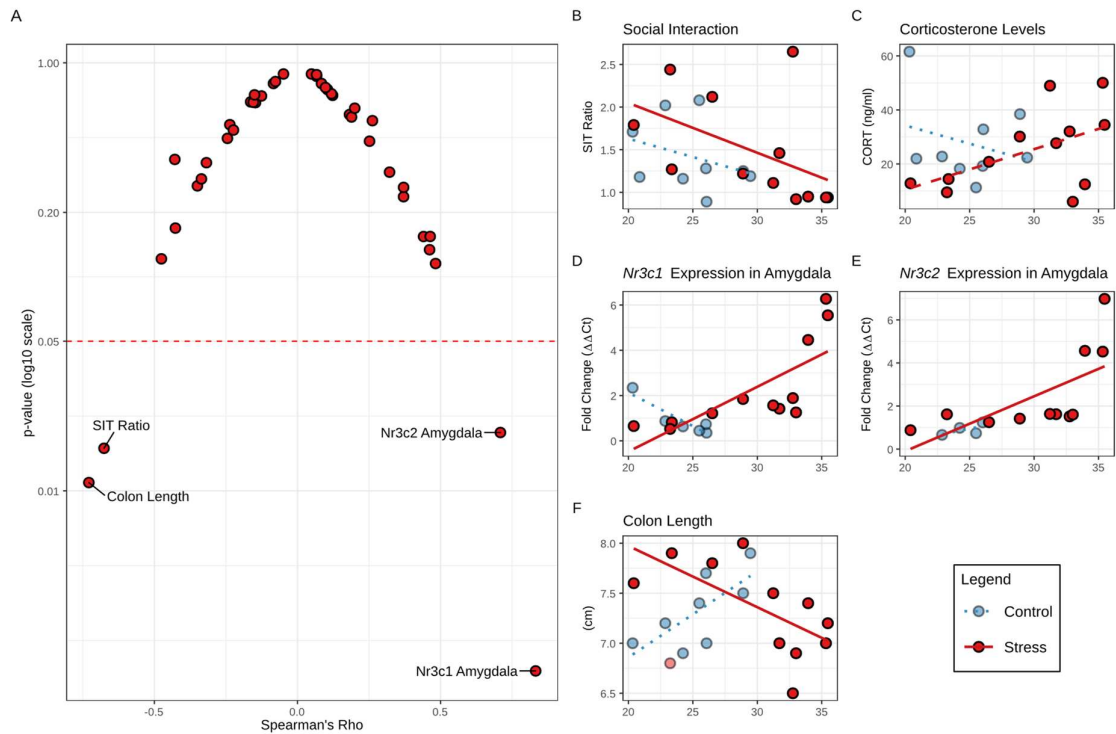


Figure 5.1-3: Volatility correlates with biological measures associated with stress. (A) Inverse volcano plot showing correlations between volatility and all other experimental parameters. Y and x-axis represent p-value on a log10 scale and spearman's rho, respectively. Red dashed line depicts $p = 0.05$. (B-F) Show individual correlations between all experimental values (y-axis) and volatility (x-axis). Measurements were assessed for reliability using Grubbs' test for outliers and jack-knifing. Controls and outliers were left out for correlations (opaque circles). Lines represent the fitted regression line, with a full line indicating a significant correlation, while a dotted line indicates no significance. Spearman: (B); $p = 0.016$, $\rho = -0.676$ (C); $p = 0.154$, $\rho = 0.441$ (D); $p = 0.001$, $\rho = 0.832$ (E); $p = 0.019$, $\rho = 0.709$ (F); $p = 0.011$, $\rho = -0.730$. Discovery cohort: Control $N = 9$; Stress: $N = 13$.

5.1.4.2 Volatility of the gut microbiome is correlated with aspects of the stress response.

Pursuing the elevated volatility in stressed animals compared to controls, we found a significant correlation between social avoidance behaviour and volatility. This finding was again replicated in the validation cohort (Figure 5.1-4).

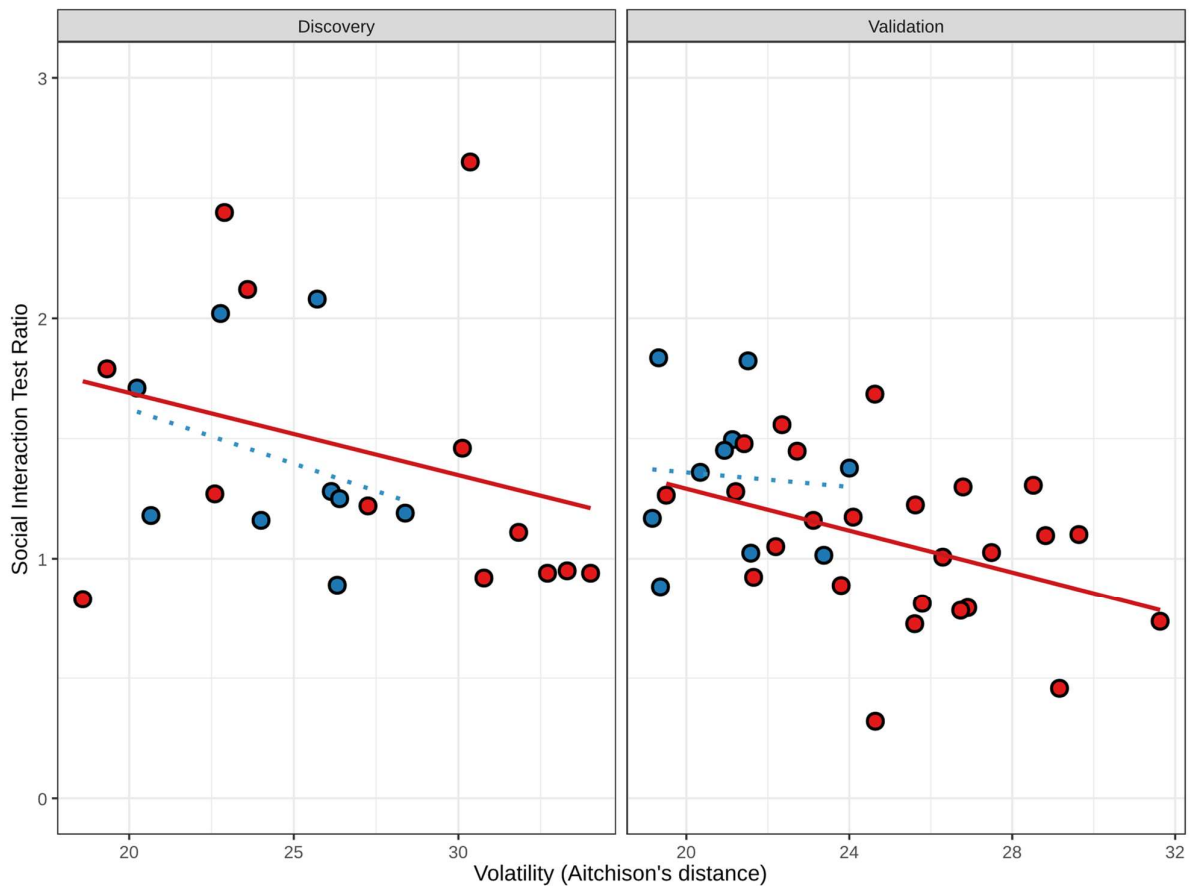


Figure 5.1-4: Microbial volatility is negatively correlated with Social Interaction Ratio after Social Defeat Stress. The x-axis shows volatility as defined by Aitchison distance moved over 10 days, while the y-axis shows the Social Interaction Ratio as defined by the ratio of time spent in proximity to an unfamiliar CD-1 mouse and time spent in the same area without the second mouse present. Red points show stressed mice, while blue points show controls. Lines represent the fitted regression line, with a full line indicating a significant correlation, while a dotted line indicates no significance. Spearman: In the Discovery cohort (Left); $p = 0.017$, $\rho = -0.68$. In the Validation cohort (Right); $p = 0.045$, $\rho = -0.41$. Discovery cohort: $N = 8$; stress: $N = 13$, validation cohort: $N = 10$; Stress: $N = 28$.

5.1.4.3 Volatility is correlated with absolute change in measures of alpha-diversity

As alpha-diversity and beta-diversity are related metrics, we asked whether changes in beta-diversity, volatility, would be related to changes in alpha diversity. We computed alpha diversity based on the first three hill-numbers; Chao1, Simpson and Shannon and found correlations between these metrics and volatility in both cohorts in the stressed mice, but never in controls (Figure 5.1-5).

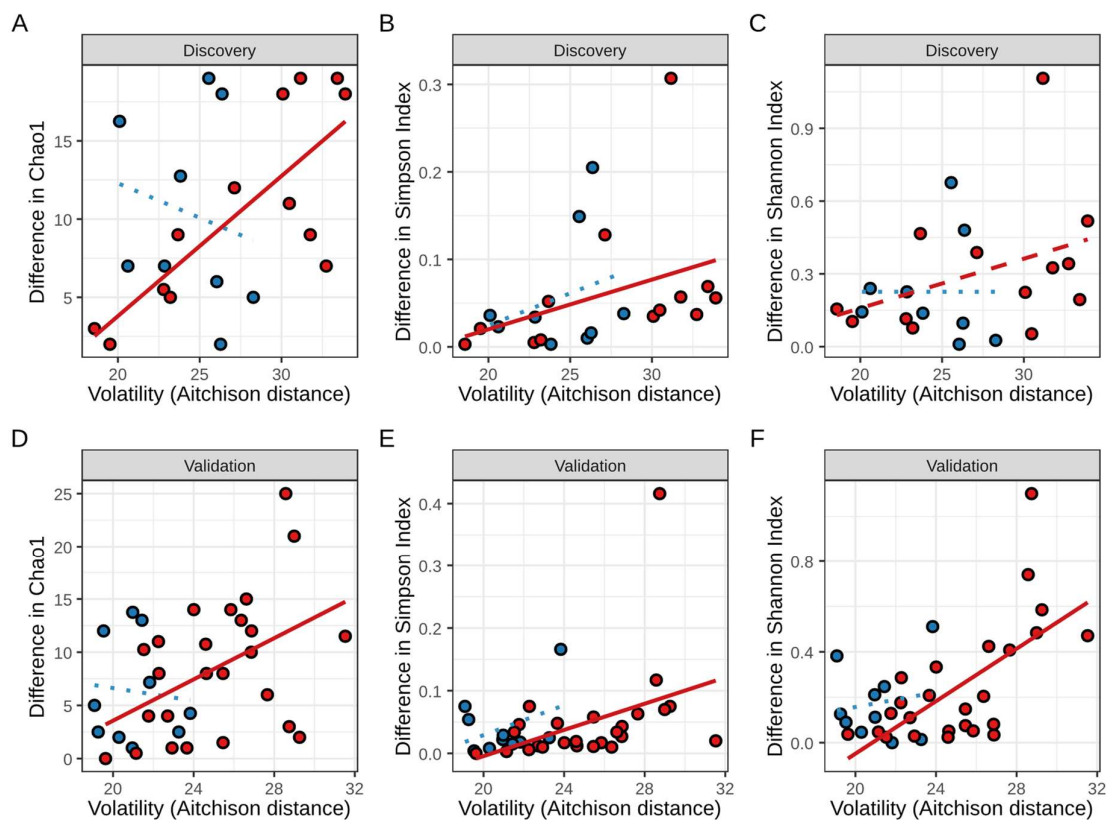


Figure 5.1-5: Microbial volatility is positively correlated with absolute changes in Alpha Diversity indices after Social Defeat Stress. The x-axis shows volatility as defined by Aitchison distance moved over 10 days, while the y-axis shows the absolute difference in Chao1 (A,D), Simpson Index (B,E) and Shannon Index (C,F) on a genus level. A-C relates to the discovery cohort, whereas D-F corresponds to the Validation cohort. Red points show stressed mice, while blue points show controls. Lines represent the fitted regression line, with a full line indicating a significant correlation, a dashed line showing a trend ($0.05 < p < 0.1$) while a dotted line indicates no significance. Spearman: (A); $p = 0.004$, $\rho = 0.739$ (B); $p = 0.014$, $\rho = 0.687$ (C); $p =$

0.10, $\rho = 0.495$ (D); $p = 0.024$, $\rho = 0.43$ (E); $p = 0.003$, $\rho = 0.55$ (F); $p = 0.002$, $\rho = 0.55$. Discovery cohort: Control $N = 9$; Stress: $N = 13$, validation Control cohort: $N = 10$; Stress: $N = 28$.

5.1.4.4 Perceived Stress in Humans

To investigate whether the relation between volatility and stress was observed in humans, we tested for correlation between volatility and stress as measured by the Perceived Stress Scale (PSS) in a cohort of students undergoing academic exams. We found a significant correlation between volatility and PSS during stress, but not under non-stress conditions I.e. before the exam period (Figure 5.1-6).

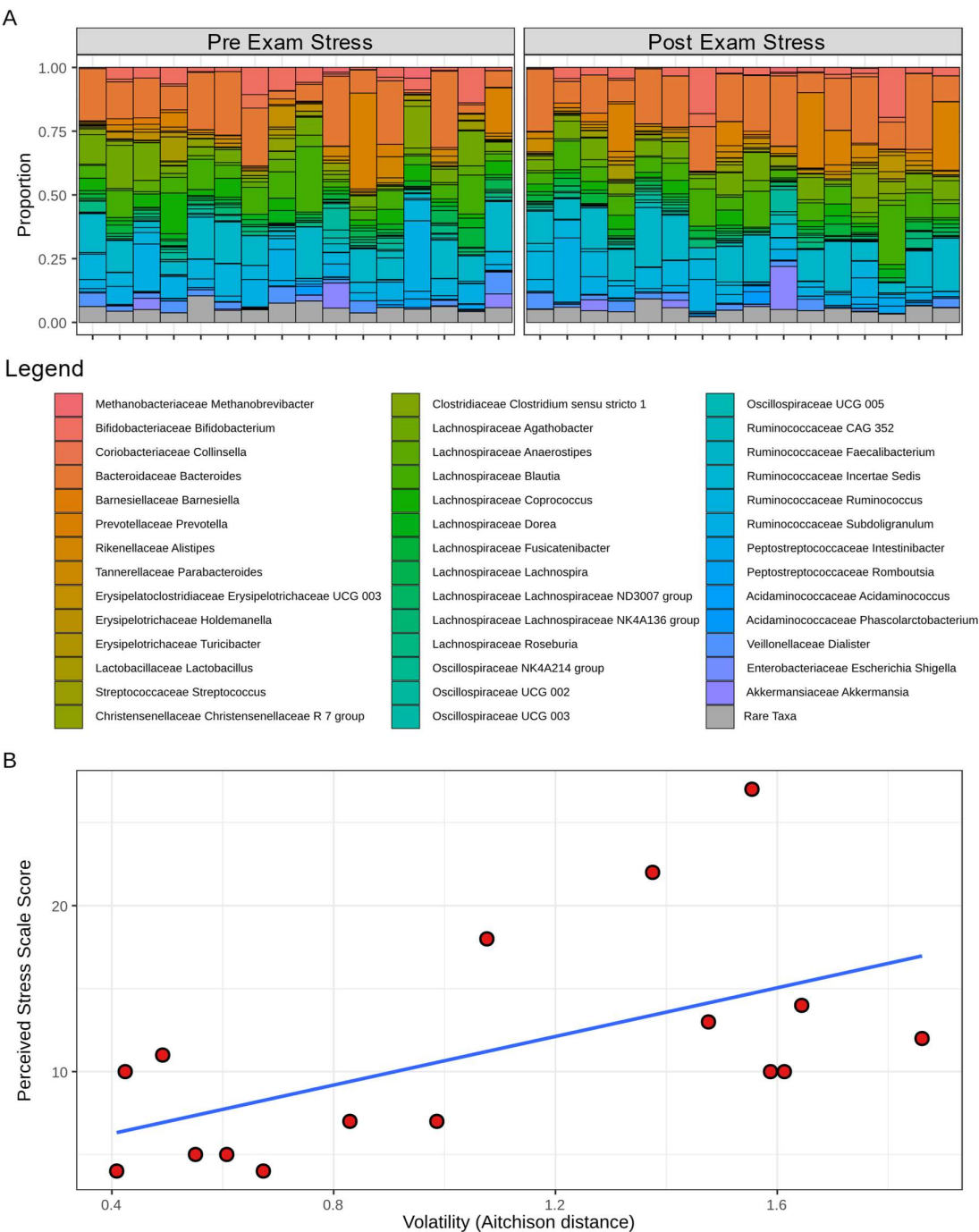


Figure 5.1-6: Microbial volatility is correlated with Perceived Stress during academic exam stress. (A) Stacked barplot showing the proportion of genera based on 16S sequences detected per sample. (B) The x-axis shows volatility as defined by Aitchison distance while the y-axis shows the Perceived Stress Scale Score during academic exam stress. Line represents the fitted regression line indicating a significant correlation. $N = 16$, Spearman: $p = 0.028$, $\rho = 0.55$.

Additionally, we set out to compare the relationship between blood cortisol and corticosterone and volatility in our human and mouse cohorts, respectively. In humans, we found a correlation between the cortisol awakening response (AUC) and volatility. Analogously, we found a significant positive correlation between evening corticosterone levels in the discovery mouse cohort as well as a trend in the same direction in the validation mouse cohort (Figure 5.1-7).

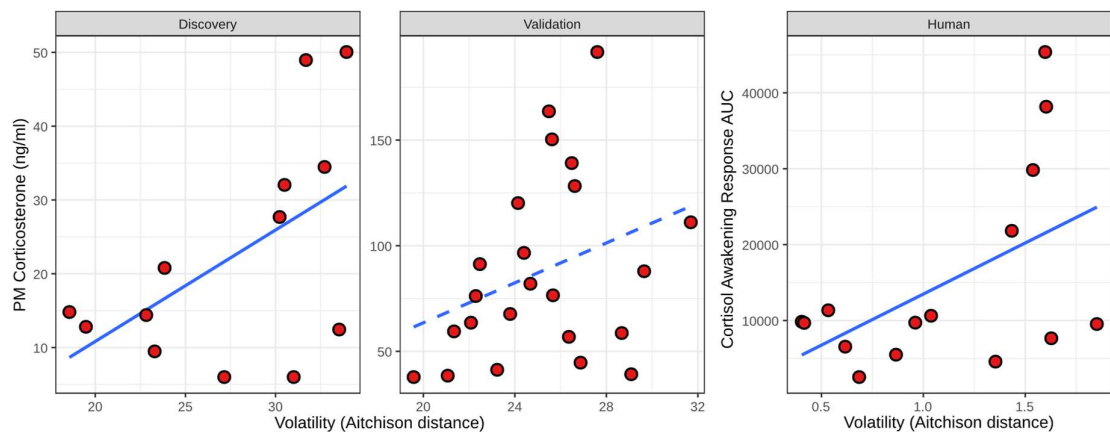


Figure 5.1-7: Microbial volatility is positively correlated with Cortisol and Corticosterone after chronic stress in humans and mice, respectively. The x-axis shows volatility as defined by Aitchison distance while the y-axis shows the evening corticosterone levels in the two left-most figures and the Cortisol Awakening Response in the rightmost figure. Line represents the fitted regression line, with a full line indicating a significant correlation and a dashed line indicating a statistical trend. Pearson: Discovery: $p = 0.0293$, $\rho = 0.537$; Validation: $p = 0.063$, $\rho = 0.327$; Human: $p = 0.024$, $\rho = 0.517$. Discovery cohort: Control: $N = 8$; Stress: $N = 13$, validation cohort: $N = 10$; Stress: $N = 28$, human cohort: $N = 16$.

5.1.4.5 Comparing Microbiome response to stress across cohorts

Finally, we set out to investigate whether other types of changes could be replicated between cohorts. We assessed differential abundance on the genus level in both discovery and validation cohorts as well as differential abundance on the level of Gut-Brain Modules (GBMs) and Gut-Metabolic Modules (GMMs). These modules represent functional pathways curated from literature that have been reported to take place in the microbiome and are involved in either gut-brain communication or in microbiome metabolism, respectively (Valles-Colomer et al., 2019). In order to compare the responses between the two cohorts, the effect sizes representing the change per microbiome feature were extracted and those modules that were present in both cohorts were tested for correlation. In the theoretical case of perfect agreement between cohorts, the rho for the correlation of the effect sizes would be 1. We found no correlation on the genus level in all animals, but in the stressed animals we found a strong positive correlation in the effect sizes on both the GBM and GMM level (Figure 5.1-8). The same procedure was then carried out comparing the functional changes in the mouse cohorts to those in the human cohort. The mouse cohorts were aggregated in order to promote interpretability (Figure 5.1-9). We observed significant correlations between the responses to chronic stress for both GBM and GMM across human and mouse host species. In particular, in terms of effect sizes, GABA synthesis and Isovaleric acid synthesis seemed to increase the most in both host species in terms of GBMs, while Mucin degradation increased the most in terms of GMMs.

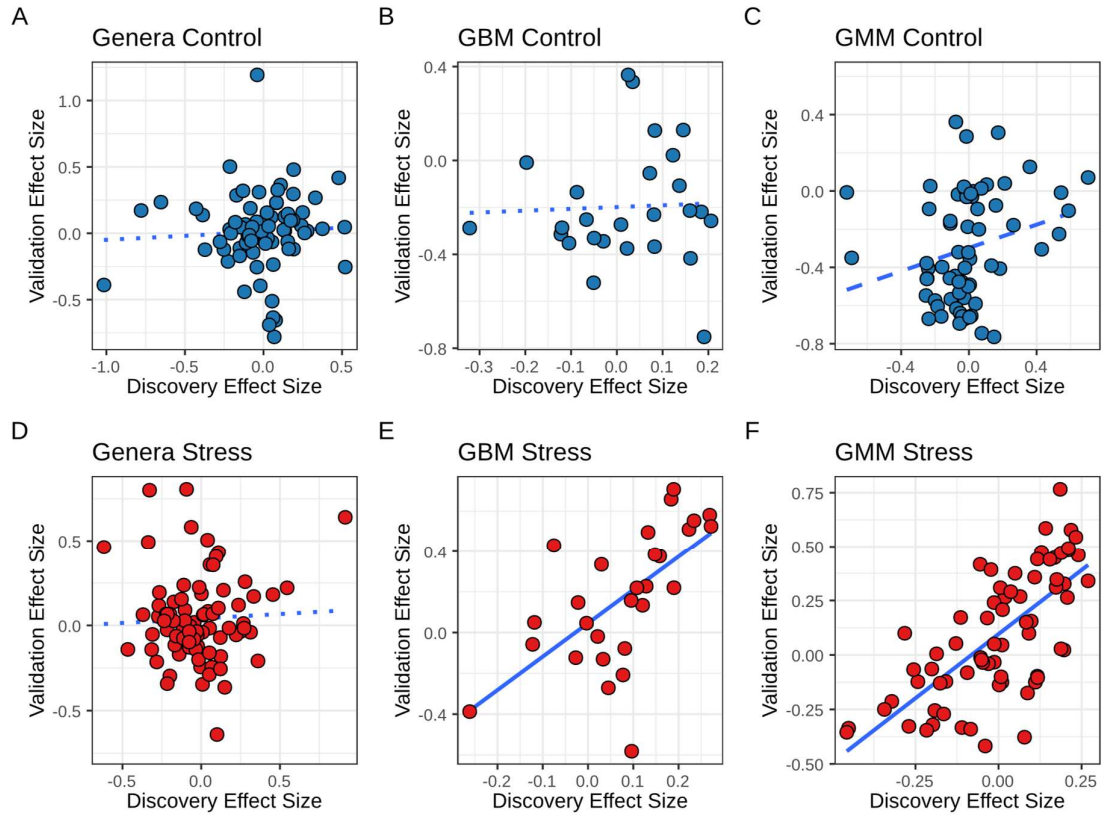


Figure 5.1-8: Microbiome responds to stress similarly on a functional level but not on a taxonomical level. The x-axis shows the effect size per feature of the Discovery cohort, while the y-axis shows the effect size per feature of the Validation cohort. Every dot is one microbial feature. The top (blue; A-C) row shows the comparisons from the Controls, while the bottom (red; D-F) shows the comparisons for the Stressed animals. Lines represent the fitted regression line, with a full line indicating a significant correlation, a dashed line showing a trend ($0.05 < p < 0.1$) while a dotted line indicates no significance. Pearson: (A); $p = 0.657$, $\rho = 0.052$ (B); $p = 0.844$, $\rho = 0.04$ (C); $p = 0.051$, $\rho = 0.243$ (D); $p = 0.674$, $\rho = 0.04$ (E); $p = 0.0004$, $\rho = 0.625$ (F); $p = 1.9 \times 10^{-11}$, $\rho = 0.694$.

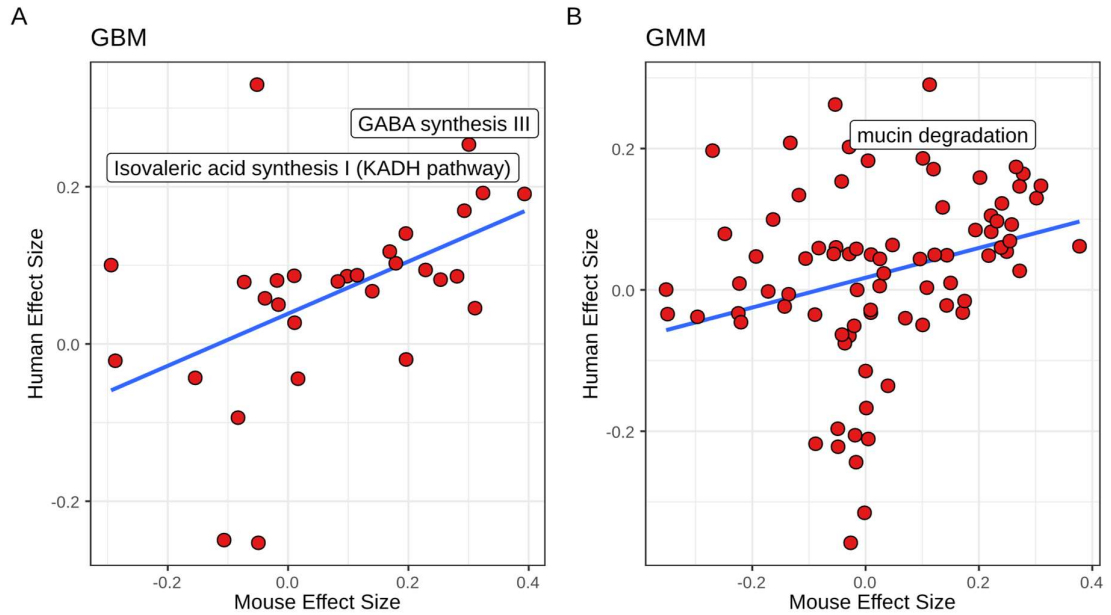


Figure 6.1-9: Microbiome responds to stress similarly on a functional level across mice and humans. The x-axis shows the effect size per feature of the Mouse cohort, while the y-axis shows the effect size per feature of the Human cohort. The left (A) figure shows Gut-Metabolic modules while the right (B) figure shows Gut-Metabolic Modules. Every dot is one microbial feature. Labels show the module names that had an absolute effect size higher than 0.2 in both mice and humans. Lines represent the fitted regression line, with a full line indicating a significant correlation. Pearson: (A); $p = 0.005$ $\rho = 0.494$ (B); $p = 0.011$, $\rho = 0.279$.

5.1.5 Discussion

Microbiome volatility is a relatively underutilized concept in microbiome ecology. With regard to stress it has not been really explored previously. One exception is in the context of irritable bowel syndrome (IBS) (Halfvarson et al., 2017), a condition which has been linked to physical and psychosocial stress exposure (Mayer et al., 2001) or Inflammatory Bowel Disease (IBD), which has also been linked to stress and anxiety (Mawdsley and Rampton, 2005), both of which found display more volatility (though it was not referred to as such) in patients compared with healthy controls (Clooney et al., 2020; Ryan et al., 2020). In this study, we further investigated the concept of volatility and, for what is to our knowledge the first time, report its potential influence on stress-related central and peripheral phenotypes.

We firstly showed that mice which had higher values in biological measures commonly associated with stress, such as changes in corticosterone levels also showed an increased volatility. Secondly, we observed a significant negative correlation between volatility and social behaviour. Notably, this correlation was found in both the discovery and validation cohorts. The implication is that severity of the stressor is related to degree of volatility, indicating volatility is related to stress susceptibility and resilience. Clearly, some stressed animals showed a higher degree of volatility than others. There are two possible explanations for this observation. The first is that volatility is determined by the microbiome, which would imply that a more volatile microbiome is a marker of stress susceptibility. Conversely, a more stable microbiome would then be a marker of stress resilience. Second, an elevated volatility after stress could be the result of a more severe stressor. While inconclusive, we initially sought to identify features in the baseline microbiome that could explain the degree of volatility after stress (data not shown), however, such differences in baseline did not hold up in the validation cohort or in the human study. Here, we were unable to find predictor features in the baseline microbiome that were generalizable over all three cohorts. In future studies, the volatility at baseline as opposed to single point measures in the current study could be measured to

address this question. Specifically, one may hypothesize that hosts with the most volatile microbiomes during neutral conditions could be the most susceptible to stress and that low volatility is a predictor of stress resilience. Additionally, future studies are needed to examine the correlation between volatility and other phenotypes of relevance following chronic stress.

The findings of this study have potential translational implications in understanding volatility in the context of human health. Indeed, the fact that we observed a correlation between self-reported stress during academic exams and volatility strengthens the notion that volatility is closely associated to stress and stress resilience. For example, one could consider volatility in the context of microbial-based interventions to treat stress-induced psychopathologies, formulations designed to stabilise the microbiome could be administered over a period of time to improve response. Alternatively, given that psychotropics are themselves known to influence the microbiome (Cusotto et al., 2019), we speculate that pre-treatment with psychobiotics which introduce specific keystone species into the microbiome, may make it more receptive to the therapeutic effects of antidepressants or anxiolytics. This latter approach could be relevant especially for patients who are resistant to treatment using conventional approaches. This also opens the door to keystone species, species that when absent will destabilize the gut ecosystem, in psychobiotic formulations. On its own, it is unclear what the impact a more volatile microbiome could be on host health, if any. One could hypothesise that volatility destabilizes the microbiome resulting in an increased susceptibility for bacterial taxa to colonize. We did not find evidence of this, but this might be due to the sanitary housing conditions of the animals and that mice are coprophagic. Indeed, fecal microbiota transplantation, representing a high-alpha-diversity pool, has been shown to expedite colonization rate over natural recovery (Suez et al., 2018). More research is warranted to test this hypothesis.

We also found consistent changes across cohorts and even between mice and humans in the microbiome after chronic stress. We did not, however, find such agreement at a taxonomic level. This is likely due to the differences in baseline microbiome between the two cohorts and the humans. Indeed, in humans, it is well-known that interpersonal variability is much lower on the functional level than on the taxonomical level (Human Microbiome Project Consortium, 2012; Mehta et al., 2018). From our findings we extrapolate that in the context of stress, while the taxonomical changes of the microbiome seem to be cohort-dependent and ultimately baseline microbiome dependent, there is a strong agreement in how the functional microbiome changes after a stressor. A stress response in the microbiome that seems invariant of the baseline condition could indicate some sort of adaptive stress response, either on the level of the microbiome or on the host level. Per definition, the GMMs and especially the GBMs have functional implications for host health. In a recent study, GBMs were shown to be influenced by diet (Valles-Colomer et al., 2019; Butler et al., 2020). Together with the finding that stress influences these modules in a specific manner, this opens up the door for psychobiotics that specifically aim to control the levels of specific modules that are known to be altered by stress. Notably, GABA synthesis was altered in both our human and mouse cohorts. The GABAergic system has been previously shown to be modulated by the microbiome in the context of the stress response (Bravo et al., 2011) and GABA-modulating bacteria have been implicated in stress-related disorders such as depression in humans (Strandwitz et al., 2019). This alteration should be pursued in future research.

To conclude, we propose that an analysis of volatility should be considered in all future longitudinal microbiome research projects. Given the novelty of this concept, we make some basic recommendations as to how examine this variable. The approach to calculating volatility presented here relies on Aitchison distance. This metric was selected because it was specifically designed to deal with compositional data, such as the microbiome (Aitchison et al., 2000; Gloor et al., 2017). While other algorithms for beta-diversity do exist, Aitchison distance has the added benefit of

satisfying the criteria for being a Euclidean distance, making comparisons between two distances within the same analysis possible. Other popular metrics like Bray-Curtis do not have this property, but rather give relative distance on a scale from zero to one, making them less suitable for the purpose of assessing volatility. We speculate that further convergence of high-dimensional mathematics, microbiology and genetics will lead to newer algorithms which prove to be more useful and easier to use. Lastly, in this study, volatility was calculated by assessing the distance 'travelled' between two points over time. Future studies should consider collecting samples over multiple time points (e.g. during stress exposure) to produce higher-dimensional geometric shapes in microbiome-space which could lead to more nuanced insights into the role of the microbiome as a mediator of the stress response.

Chapter 6 Human Microbiome-Gut-Brain Axis Studies

6.1 A specific dietary fibre supplementation improves cognitive performance—an exploratory randomised, placebo-controlled, crossover study

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6.1.1 Abstract

6.1.1.1 Rationale:

The impact of the microbiota on the gut-brain-axis is increasingly appreciated. A growing body of literature demonstrates that use of dietary fibre and prebiotics can manipulate the microbiota and affect host health. However, the influence on cognition and acute stress response is less well understood.

6.1.1.2 Objectives:

The objective of this study was to investigate the efficacy of a dietary fibre, polydextrose (PDX), in improving cognitive performance and acute stress responses through manipulation of the gut microbiota in a healthy population.

6.1.1.3 Methods:

In this double-blind, randomised, placebo-controlled, cross-over design study, 18 healthy female participants received 12.5g Litesse®Ultra (>90% PDX polymer) or maltodextrin for four-weeks. Cognitive performance, mood, acute stress responses, microbiota composition, and inflammatory markers were assessed pre- and post-intervention.

6.1.1.4 Results:

PDX improved cognitive flexibility as evidenced by the decrease in the number of errors made in the Intra-Extra Dimensional Set Shift (IED) task. A better performance in sustained attention was observed through higher number of correct responses and rejections in the Rapid Visual Information Processing (RVP) task. Although there was no change in microbial diversity, abundance of *Ruminiclostridium 5* significantly increased after PDX supplementation compared to placebo. PDX supplementation attenuated the increase of adhesion receptor CD62L on classical monocytes observed in the placebo group.

6.1.1.5 Conclusions:

Supplementation with the PDX resulted in a modest improvement in cognitive performance and inflammatory profile. The results indicate that PDX could benefit gut-to-brain communication and modulate behavioural responses.

6.1.1.6 Introduction

There is a growing realisation that the symbiotic relationship between microbes and their host can significantly impact on health. The gut microbiota is vital for normal physiological functioning, aiding in the digestion and utilization of nutrients, development of the immune system, and general host metabolism (Jandhyala et al., 2015). Many diseases (e.g., inflammatory bowel disease (Sheehan et al., 2015), obesity (Martinez et al., 2017) or autism spectrum disorder (Strati et al., 2017; Berding and Donovan, 2018)) have now been associated with an altered gut microbial profile, often manifested by a decreased abundance of beneficial or increased abundance of pathogenic bacteria, or a reduced microbial diversity. Besides the potential involvement in psychological disease processes, an ever-increasing number of studies have shown that stress, anxiety, memory, cognition and neuroinflammation may be effected by the composition of the gut microbiota (Rea et al., 2016; Allen et al., 2017; Sarkar et al., 2018; Smith and Wissel, 2019). Therefore, modulation of the gut microbiota is emerging as an exciting potential strategy to support mental health and cognitive function (Dinan et al., 2019; Long-Smith et al., 2020). In this context, the term psychobiotic was coined to describe any exogenous influence (i.e., probiotics, prebiotics, dietary fibre) whose positive effect on mental health is bacterially mediated (Dinan et al., 2013; Sarkar et al., 2016).

The use of dietary fibre, which includes most prebiotics, has been an exciting opportunity to support the growth of beneficial host microbiota (Gibson et al., 1995; Bindels et al., 2015; Holscher, 2017). Indeed, some prebiotics fibres such as inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and polydextrose (PDX), which are resistant to digestion and absorption in the intestinal tract, have been shown to beneficially alter the microbiota composition and promote the growth of beneficial bacteria such as *Bifidobacterium* (Roytjo and Ouwehand, 2014; Holscher, 2017). Systemically, improvements in metabolic outcomes and increased satiety (Hull et al., 2012) in an obese population after supplementation with PDX in combination with probiotic strains were described (Hibberd et al., 2019). While pre-clinical studies have shown potential benefits for prebiotic fibres in brain health

(Savignac et al., 2013; Tarr et al., 2015; Burokas et al., 2017; Boehme et al., 2019), there are limited studies published on the effects on brain and cognitive function in humans. A small-scale study in healthy individuals has demonstrated that intervention with B-GOS resulted in an improved stress response as well as emotional information processing (Schmidt et al., 2015). Improvements in memory tasks and subjective improvements in mood were observed in a study with healthy individuals receiving a prebiotic of oligofructose-enriched inulin (Smith et al., 2015), while a B-GOS formulation significantly improved anxiety levels in patients with irritable bowel syndrome (Silk et al., 2009). However, the impact of PDX on stress responses and cognitive performance has not been investigated. Therefore, in this study, we examined the potential of PDX (Litesse®Ultra, Danisco USA Inc., Terre Haute, IN, USA) to modulate the microbiota composition, mood, cognition and acute stress responsivity. We hypothesized that PDX supplementation would increase the abundance of beneficial bacteria, positively modulate cognitive performance and decrease physiological and psychological response to stress.

6.1.2 Methods

This research study was conducted in accordance to the Good Clinical Practice guidelines and the Declaration of Helsinki. The protocol was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Protocol Number APC076). Informed consent was obtained from all participants prior to enrolment into the study.

6.1.2.1 Participants and Study Design

This study was designed as double-blind, randomised, placebo-controlled, using a repeated measure cross-over design study. Due to the higher prevalence of stress-related conditions such as depression in females, female participants between the ages of 18 and 40 were recruited in the Cork area and completed the study between March 2018 and December 2018. Eligible participants were randomised to take 12.5g of Litesse®Ultra (>90%PDX polymer, Danisco USA Inc., Terre Haute, IN, USA) or

placebo (maltodextrin; matched for taste, colour, odour) for 4 weeks, followed by a 4-week washout period and a 4-week period of PDX or placebo (**Figure 6.1-1a**). The same dose of 12.5g per day of Litesse®Ultra was selected for this study as it closely resembles the efficacious doses used in previous studies for metabolic health (Stenman et al., 2016; Ibarra et al., 2017). Participants were instructed to consume one sachet of blinded study product every morning mixed in a hot drink. Reminders to consume the study product were sent to participant 3 times weekly. Additionally, participants were instructed to refrain from introducing other probiotic or nutrition supplements during the course of the intervention and keep dietary intake and physical activity consistent throughout the study.

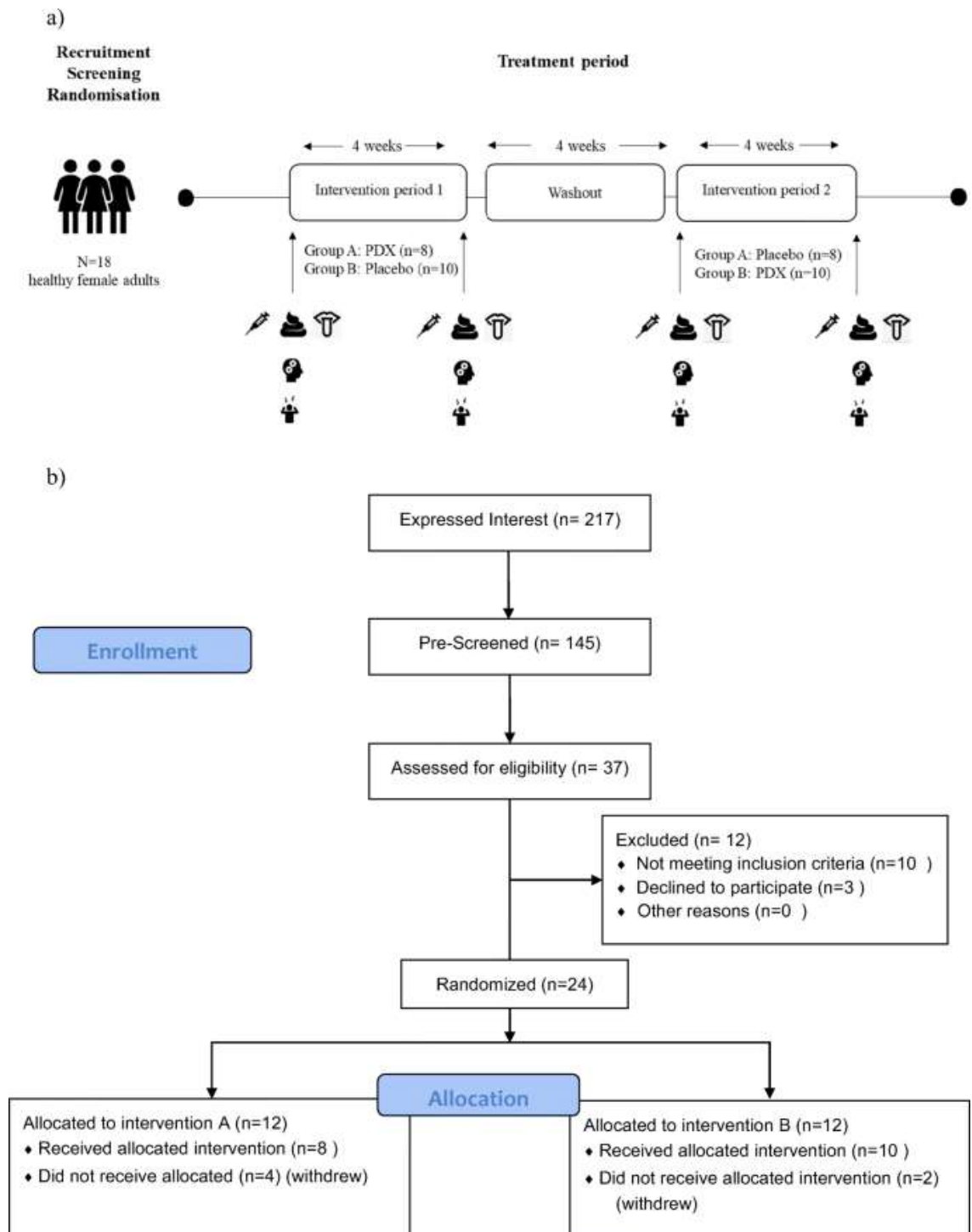


Fig. 6.1-1 Study visit diagram illustrates study procedures over the 12-week study period (a). Intervention periods were separated by a 4-week washout period. Biological samples (stool, saliva, blood), cognitive performance (CANTAB), and acute stressor (SECPT) were collected and assessed pre- and post-intervention periods. Participants were block randomized (block of 4) with an allocation ratio to group/treatment of 1:1 of PDX:placebo to account for balanced group allocation. In intervention period 1, group A received PDX and group B received placebo. In

intervention period 2, group A received placebo and group B received PDX. b CONSORT diagram shows subject enrolment and allocation to treatment groups.

Participants were excluded from the study if they had any significant acute or chronic illness, were taking medication, were peri-menopausal, menopausal or post-menopausal, were pregnant, or lactating, were not fluent in the English language, were colour blind, had dyslexia or dyscalculia, were vegan, a habitual daily smoker or had taken any pro-, pre- or antibiotic 4-weeks prior to enrolment in the study.

At the initial screening visit, participants were screened for any psychiatric disorder using the MINI International Neuropsychiatric Interview (MINI) and demographic data were collected. Additionally, a battery of self-reported questionnaires to obtain a baseline psychological profile were completed (for description of test see Supplementary Methods). Participants also completed the National Adult Reading Test (NART, (Nelson and Willison, 1991) as a brief measure of verbal IQ. Cognitive assessment and the acute stressor procedure as well as collection of biological samples and questionnaire data (as described below) were completed at visits 2, 3, 4 and 5. All study visits were performed in the HRB Clinical Research Facility at University College Cork.

Participants, study facilitators, nurses and research analysts were kept blinded until all data analysis was completed. The block randomisation (block of 4) of treatment schedules was carried out by DuPont. To balance group assignment, an allocation ratio to group/treatment of 1:1 PDX:placebo was used. Remaining study product was collected from participants to check for compliance following visit 3 and 5.

6.1.2.2 Neuropsychological assessment

The Cambridge Neuropsychological Test Automated Battery (CANTAB) was used to assess cognitive performance (<https://www.cambridgecognition.com/cantab/>). The test battery was presented on a touch screen monitor. A researcher provided verbal instructions from a standardized script as well as specific verbal prompts or encouragement when needed. To avoid effects of fatigue for tests completed later

in the session, the tests were presented in different orders for different participants, using a Latin square design. The whole battery lasted approximately 40 minutes and included the Motor Screening Test (MOT), Rapid Visual Information Processing (RVP), Paired Associates Learning (PAL), Spatial Span (SS), the Intra-Extra Dimensional Set Shift (IED) and the Emotion Recognition Task (ERT). A detailed description of each task can be found in the **Supplementary Methods**.

6.1.2.3 Faecal Sample Collection and 16S rRNA sequencing

Freshly voided faecal samples were collected from study participants into plastic containers containing an Anaerogen sachet (Oxoid AGS Anaerogen Compact, Fischer Scientific, Dublin), and kept cool in a refrigerator until delivery to the laboratory. The sample was aliquoted and stored at -80° for later analysis. Participants were instructed to collect the faecal sample as close to the study visit as possible. The range of times between sample produced by the participant and freezing in the laboratory varied between 4 to 6 hours. The samples were stored in the participant's fridge until transport to the study site using a cool pack. Upon arrival at the study site the sample was placed in the fridge until collected by the researcher processing the sample.

6.1.2.4 DNA Extraction:

DNA was extracted from 250mg faecal samples using a previously described modified protocol, which combined the repeat bead beating method with the QIAmp Fast DNA Stool Mini Kit (Qiagen, Germany) (Yu and Morrison, 2004; Fouhy et al., 2015). DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences Dublin, Ireland or Life Technologies or Thermo Fisher Scientific) and the Qubit® dsDNA HS Assay Kit (Bio-Sciences Dublin, Ireland or Life Technologies or Thermo Fisher Scientific), along with being run on a 1.5% agarose gel to check the DNA quality. Extracted DNA was then stored at -20°C until prepared for 16s rRNA sequencing.

6.1.2.5 16S rRNA Microbiome Sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified from the DNA extracts and prepared for sequencing using the Illumina 16S Metagenomic Sequencing Library Protocol (Amplicon et al., 2013). A detailed description of the 16S rRNA sequencing protocol can be found in the **Supplementary Methods**.

6.1.2.6 Blood Inflammatory Profile

Whole blood was collected into 4 mL lithium-heparin containing tubes (Greiner Bio-One, 454029). Plasma samples were collected into 3 mL K3EDTA tubes (Cruinn Diagnostics Limited, Dublin). Blood samples to analyse the impact of the acute stressor on inflammatory markers were taken immediately upon completion of the stressor (described below). Plasma samples were centrifuged at 1500g for 10 minutes. The supernatant was aliquoted and stored at -80°C for later analysis.

6.1.2.7 Whole blood stimulation

Whole blood was diluted 1:10 with Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma Aldrich) supplemented with 10% foetal calf serum and 5% penicillin streptomycin. Of the diluted mixture, 500µL was aliquoted to a 24-well plate. Each well was stimulated with 5 µL of the stimulant (TLR4 agonist - E. coli K12 LPS and TLR5 agonist – *S. typhimurium* Flagellin, Invivogen, Toulouse, France). The blood was harvested after 24 hours of incubation at 37°C and stored at -80°C for later analysis.

6.1.2.8 Cytokine quantification

Cytokine levels from plasma samples and stimulated blood samples were quantified using the V-PLEX Proinflammatory Panel 1 (human) Kit (MSD, K15049D). Cytokine quantification was done according to the manufacturer's guidelines with one modification, where 100 µl sample was added directly onto the plate without dilution.

6.1.2.9 Flow cytometry

Mononuclear cells were isolated using Ficoll® Paque Plus (Sigma-Aldrich, GE17-1440-02) from whole blood. Cells were counted and 2×10^6 cells were used for flow cytometric staining. Samples were first treated with BD Horizon™ Fixable Viability Stain 780 (BD Biosciences, 565388), after which cells were incubated with FcR blocking reagent (Miltenyi Biotec, 130-059-901) and a mix of extracellular antibodies (**Supplemental Table 1**) in Brilliant Stain Buffer (BD Biosciences, 563794). Cells were finally fixed with 4% paraformaldehyde. Samples were analysed the following day using a BD FACSCelesta (BD Biosciences). Data were analysed using FlowJo (version 10) for the gating strategy of classical monocytes (**Supplemental Figure 2**). Briefly, cells were first selected based on FSC/SSC, after which doublets were excluded. Live cells (FVS780-) were selected, after which SSC^{high} cells (granulocytes) and DUMP- cells (CD3+ T cells, CD19+ B cells, and CD56+ NK cells) were excluded. Classical monocytes (CD14+, CD16-) were subsequently selected based on CD14 and CD16 receptor expression. Cell numbers were normalized to total live single-cell numbers. Receptor expression was assessed using median fluorescent intensity (MFI). Details on the flow cytometry method can be found in the **Supplementary Methods**.

6.1.2.10 Acute Stress Response

The socially evaluated cold pressor test (SECPT) (Schwabe et al., 2008) combining a psychological with a physiological stressor was utilized to elicit an acute stress response as previously described (Allen et al., 2016). Galvanic skin response was measured during the stress using the NeXus 4 Software (Biotrace, Mind Media, Netherlands). A total of eight saliva samples were taken during the stressor, with the first sample taken 5 minutes before the beginning of the stressor.

6.1.2.11 Cortisol Awakening Response

Morning saliva samples were collected using the salivette system (Sarstedt, Germany). Participants were instructed to collect the saliva samples in the morning of the day of their study visit. A total of four samples was collected, with the first one collected upon waking, the second one 30 minutes later, and the third and fourth one in 15 minutes increments. Participants were instructed to not brush their teeth until after all saliva samples were collected, to not eat or drink anything prior to the first sample, and to avoid eating and drinking 15 minutes prior to the remaining samples. Saliva samples were centrifuged at 1500g for 5 minutes, aliquoted and stored at -80°C for later analysis.

6.1.2.12 Cortisol analysis

Salivary cortisol (CAR and SECPT saliva) was analysed in duplicates using the ENZO Life Sciences enzyme-linked immunosorbent assay (ELISA) kits (Exeter, UK) per manufacturer's instructions. The lower limit of detection was 0.156 nmol/L. Inter- and intra-assay coefficients of variability were 13.4% and 10.5%.

6.1.2.13 Self-report questionnaires during study visits

Participants completed self-reported paper-based questionnaires to assess perceived stress (Cohen's Perceived Stress Scale (PSS) (Cohen et al., 1983)), depression and anxiety levels (Beck's Depression Inventory II (BDI-II) (Beck et al., 1996)), Hospital Anxiety and Depression Scale (HADS-A and HADS-D) (Bjelland et al., 2002)) and psychopathological symptoms (Symptom Checklist-90-R (SCL-90-R) (Derogatis and Unger, 2010)). Dietary intake was quantified using a food frequency questionnaire (FFQ, (Harrington et al., 2011)). Gastrointestinal (GI) tolerability was assessed using a GI symptom visual analogue scale (VAS). Changes in stool type were captured by the Bristol Stool chart.

6.1.2.14 Statistical Analysis

All data were analysed using SPSS 25 (IBM, Armonk, NY, USA). All data analysis was performed using the intention-to-treat analysis, and the last observation carried forward approach was employed for missing data. Standardized z-scores (using a cut off value of ± 3.29) and box plots were used to identify outliers.

Data were examined for normality using the Shapiro-Wilk statistic and log transformed, if necessary. Differences between outcome measures between pre-and post-intervention were analysed using within-subject repeated measured analysis of variance (ANOVA) and *post-hoc* paired samples t-test. Where parametric assumptions were violated and data transformation did not achieve normal distribution, the Friedman and paired sample Wilcoxon signed-rank tests were applied. Areas under the curve for cortisol measurements were calculated with respect to ground (AUC_G) (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). Flow cytometry data were assessed using the paired-sample Wilcoxon signed-rank test or t-test (differences between pre- and post-stress) or Mann-Whitney U test (differences between the two treatment groups). Categorical data (i.e., Bristol stool chart) were analysed using the Chi-squared test. Correlation between microbial abundance and other outcome measures were performed using spearman rank correlation. $P < 0.05$ was considered significant and $p < 0.1$ was considered a trend. Data is expressed as mean \pm SEM.

6.1.2.15 Bioinformatics analysis:

Three hundred base pair paired-end reads were prefiltered based on a quality score threshold of > 28 and trimmed, filtered for quality and chimeras using the DADA2 library in R (version 3.6.1) (Callahan et al., 2016). Samples with a quality score < 28 and/or < 10000 reads were discarded, resulting in a range of reads for the kept samples was 10696 – 143246. Taxonomy was assigned with DADA2 against the SILVA SSURef database release v132. Unless mentioned otherwise, recommended parameters according to the DADA2 manual were adhered to. A total of 1353 amplicon sequence variants (ASVs) were identified. ASVs were summarized on a genus level, with ASVs that were unknown on the genus level not being considered

in downstream analysis, as were genera that were only detected as non-zero in 5% or fewer of total samples.

Microbiome data-handling was done in R with the Rstudio GUI (version 1.2.1555). Principal component analysis (PCA) was performed on centre-log ratio transformed (clr) values using the ALDEx2 library (Fernandes et al., 2013). Number of permutations was always set to 1000. Differential abundance of microbes between groups was assessed using the ALDEx2 library. As part of testing for correlations between microbial abundance and metadata, skadi, an implementation of jackknifing and Grubb's test, was used to assess the reliability of the data and detect outliers (R scripts available online, <https://github.com/thomazbastiaanssen/Tjazi> (DOI: <https://doi.org/10.5281/zenodo.1480804>). Correlation was assessed using Spearman's rank correlation coefficient. For datasets in which the condition of normality was violated the non-parametric Kruskal-Wallis test was used and post-hoc analysis was done using the Wilcoxon test. A p-value of < 0.05 was deemed significant in all cases. To correct for multiple testing, the Q-value post-hoc procedure was performed with a q-value of 0.1 as a cut-off (Storey et al., 2015).

6.1.3 Results

A total of 18 healthy female participants completed the study. The CONSORT diagram is shown in **Figure 6.1-1 b**. Baseline demographics can be found in **Table 1**. Participants were of good health, had a normal BMI (mean: 23.7) and baseline psychological profile and were free of gastrointestinal symptoms or disorders.

6.1.3.1 Neurocognitive performance

In the CANTAB battery, an improvement in IED (cognitive flexibility) and RVP (sustained attention) after PDX supplementation was observed.

In the IED task, there was a significant interaction effect between time (pre- vs. post-intervention) and product (PDX vs. placebo) for completed stage errors ($F(1,16) = 5.8$, $p=0.03$) and completed total trials $F(1,15) = 9.6$, $p=0.007$) (**Figure 6.1-2 a,b**). Post-hoc analysis revealed that compared with the placebo phase, participants made less errors on the stages successfully completed (completed stage errors, $t(1,17) = -3.9$, $p=0.001$; 95% CI [-2.65, -0.79]) and successfully completed the stages using a lower number of trials (completed stage trials, $t(1,17) = -4.9$, $p<0.001$; 95%CI [-3.66, -4.95]) after the PDX phase.

Similarly, improvements in the number of correct responses, total correct rejections and failure to respond in the RVP task after PDX supplementation were recorded (**Figure 6.1-2 c-e**). The number of correct responses (probability of hit ($t(1,17) = -3.5$, $p=0.003$; 95%CI [-0.18, -0.044]) and total correct rejections ($t(1,17) = -3.9$, $p= 0.001$; 95%CI [-10.98,-3.24]) increased after the PDX but not placebo condition. There was also a decrease in failure to respond (total misses ($t(1,17) = 3.4$, $p=0.003$; 95%CI [1.17, 4.83]) after PDX supplementation. However, neither of these outcome measures were significantly different between treatments.

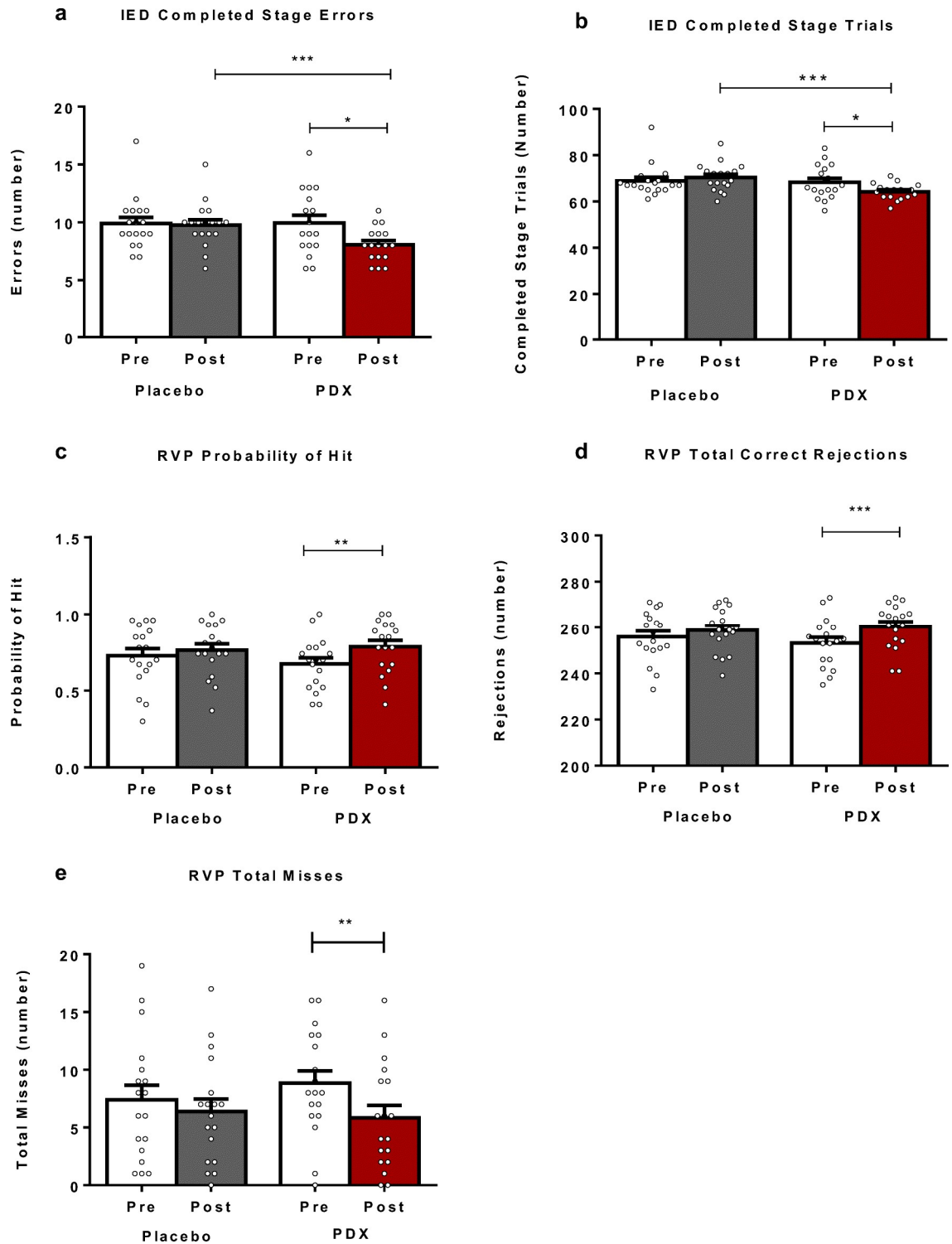


Fig. 6.1-2 PDX supplementation improved neurocognitive performance in the IED and RVP task of the CANTAB battery. After PDX supplementation, in the IED task, participants made less errors on stages successfully completed (a) and had a lower number of trials completed on all attempted stages (b). In the RVP task, PDX increased the number of correct responses (c), and total correct rejections (d) and lowered the number of misses (e). Individual dots represent a participant; data shown as mean \pm

*SEM; data was analysed by repeated measures ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; IED, intra extra dimensional set shift; RVP, rapid visual information processing*

6.1.3.2 Microbiota composition

All DNA quantity was above 8.3 ng/ul which is the recommended threshold to pool the DNA to for sequencing. Single bright bands of the expected fragment size were seen for all the samples on the agarose gel.

A boxplot of the relative abundance of microbiota composition at the genus level pre- and post- placebo and PDX intervention is shown in **Figure 6.1-3 a**. A total of 168 genera were identified through 16S rRNA sequencing in this cohort. Undirected pairwise analysis (Wilcoxon rank sum test with storey's q-value post-hoc correction) revealed significant changes in only one ASV after PDX intervention. A significant increase to the genus *Ruminoclostridium* 5 ($q=0.002$; effect size: 1.48) only after PDX, but not placebo supplementation, was observed (**Figure 6.1-3 b**).

Microbial α -diversity within each participant quantified before and after each treatment course did not differ between treatments (Chao 1, $p=0.85$; Simpson, $p=1.0$; Shannon, $p=1.0$; data not shown). Likewise, no statistically significant differences in β -diversity were detected ($p=0.996$; data not shown).

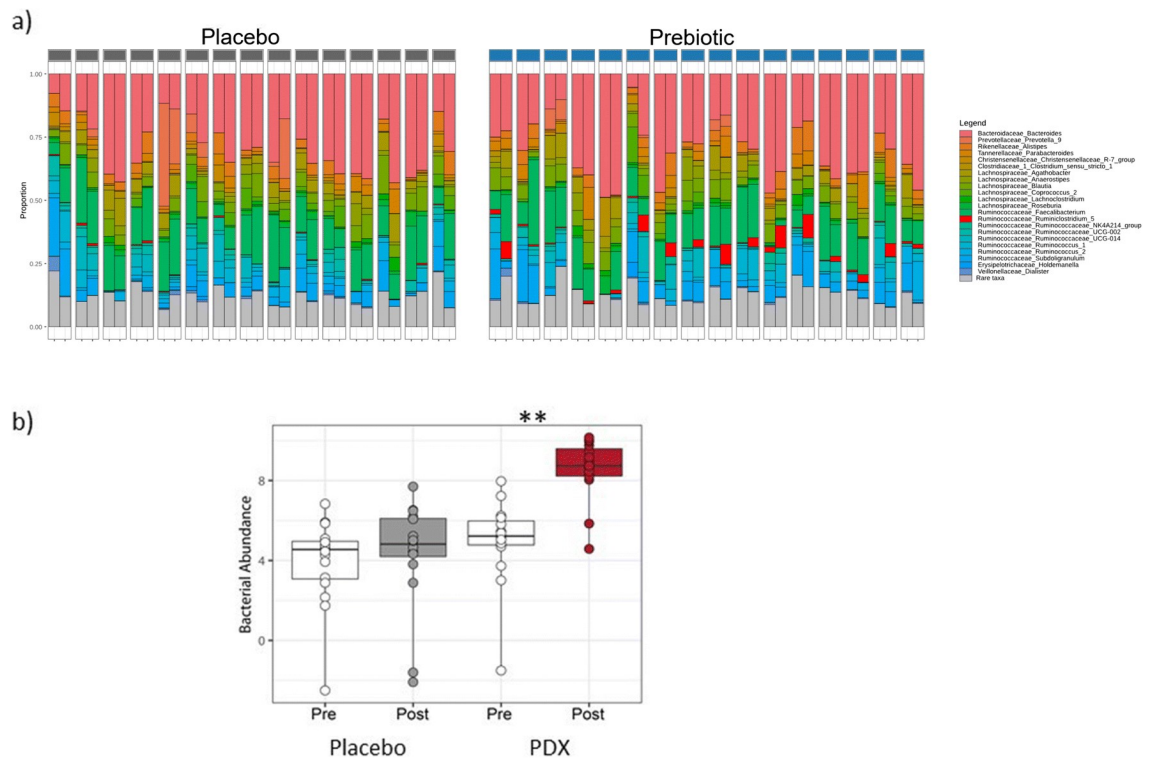


Fig. 6.1-3 Abundance of *Ruminiclostridium 5* increased after PDX supplementation. Representative graphs for changes in microbiota composition pre- and post-placebo and PDX supplementation. a Stacked bar plots showing abundance of microbes at genus level for each participants pre (left) and post (right) treatment arm. Each pair of bars represents one individual. b A significant increase in *Ruminiclostridium 5* after PDX but not placebo supplementation was observed. Data was analysed by Kruskal-Wallis and paired-sample Wilcoxon signed-rank tests; $^{**}q < 0.01$; data in (b) is expressed as median (horizontal line), interquartile range (box), and range (whiskers)

6.1.3.3 Inflammatory profile

Flow cytometric analysis revealed that acute stress did not influence circulating classical monocyte (CD14+, CD16-) levels (**Figure 6.1-4 a,b**). Acute stress did increase the expression of the adhesion receptor CD62L on classical monocytes in the placebo group, but not in participants that received PDX supplementation ($Z = -2.803$, $p = 0.005$) (**Figure 6.1-4 c**). This increase in CD62L expression in the placebo group was significantly higher compared to the PDX group ($U = 14$, $p = 0.005$) (**Figure 6.1-4 d**).

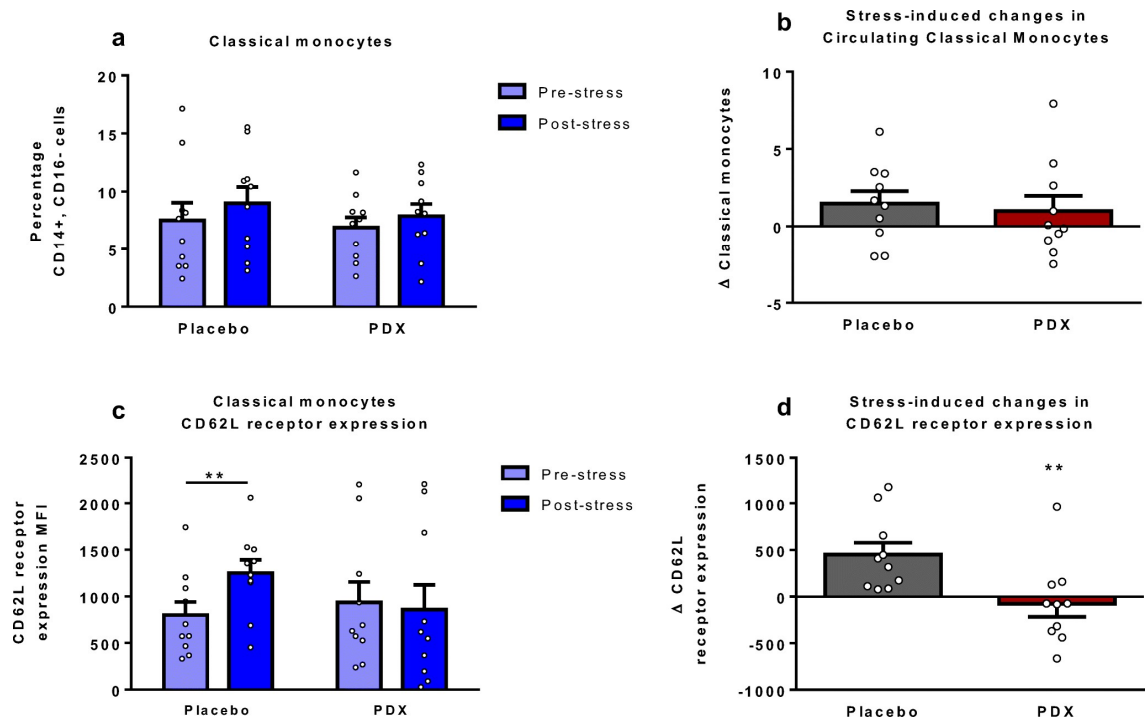


Fig. 6.1-4 PDX supplementation lowers acute stress-induced increases in CD62L receptor expression on classical monocytes. Flow cytometric analysis was performed to quantify classical monocytes (CD14⁺, CD16⁻) (a, b), and their expression of the CD62L receptor (median fluorescent intensity (MFI)) (c, d). Individual dots represent participants (n = 10/group). **p < 0.01; data expressed as mean ± SEM; data was analysed using paired-sample Wilcoxon signed-rank test or t test (differences between pre- and post-stress) or Mann-Whitney U test (differences between the two treatment groups)

There was no difference in the inflammatory profile of plasma samples (**Table 2**) or in the TLR4 stimulated whole bloods (**Table 3**). In the TLR5 stimulated bloods, a trend for lower concentrations of IFN γ (t = -1.85, p = 0.087; 95%CI [-24.4, 1.89]) and IL-2 (t=-1.9, p= 0.074; 95% CI [-3.46, 0.18]) post- PDX supplementation compared to placebo was observed (**Table 3**).

6.1.3.4 Stress responses

Neither cortisol awakening response or salivary cortisol concentrations after the acute stressor were affected by PDX supplementation (**Figure 6.1-5**). Likewise, the

galvanic skin response was not affected by the stressor or PDX administration (data not shown).

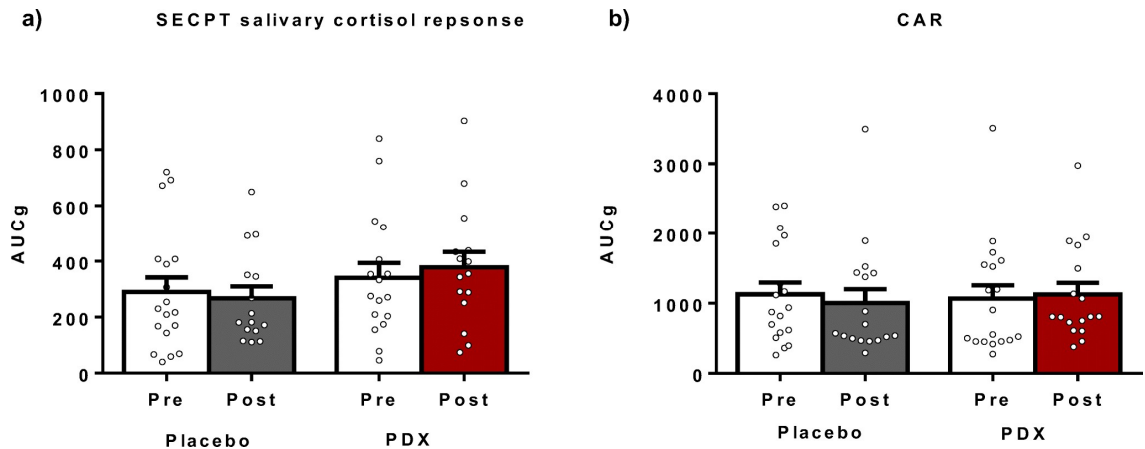


Fig. 6.1-5 Salivary cortisol responses to acute stressor and morning awakening response did not change after PDX supplementation. Salivary cortisol for each visit as measured by the area under the curve with respect to ground (AUCg). Neither total salivary output in response to the socially evaluated cold pressor test (SECPT, (a)), nor cortisol awakening responses (CAR, b) were affected by PDX supplementation. Data analysed by Friedman test; data expressed as mean \pm SEM

6.1.3.5 Mood and Psychological Symptoms

PDX supplementation did not affect levels of anxiety (HADS-A score), depression (HADS-D score, BDI-II score), perceived stress (PSS score) or psychopathological symptoms (SCL-90-R global severity index) (**Table 4**).

6.1.3.6 Dietary intake

Intake of total calories, macronutrient and dietary fibre intake as assessed by the FFQ was mostly consistent throughout the study period. There was a slight decrease in vitamin E ($Z=-2.4$, $p=0.02$), polyunsaturated fatty acids ($Z=-2.4$, $p=0.02$) and monounsaturated fatty acids ($Z=-1.7$, $p=0.09$) during the PDX period (**Supplemental Table 2**).

6.1.3.7 Compliance and tolerability

Overall, participants showed good compliance to study product (>70%). One participant showed lower compliance at 60% during the PDX phase. No treatment related adverse events occurred during the PDX treatment arm.

Overall, PDX supplementation was well tolerated as measured by the GI VAS at each visit. Only two people reported GI symptoms after PDX supplementation as assessed by the GI VAS, of which one reported abdominal pain. However, these symptoms were only present one out of every 10 days. Three participants reported development of bloating after the PDX intervention. However, these participants also reported bloating at other time points of the study, suggesting that bloating might be due to other causes.

Generally, satisfaction with bowel habits was very high and GI symptoms did not interfere with life satisfaction in this cohort. PDX supplementation did not change GI symptom satisfaction and GI symptom life interference. Stool consistency as measured by the Bristol Stool chart did not change throughout the study period (mean Type 3 (“Like a sausage but with cracks on its surface”)) (**Supplemental Table 3**).

6.1.4 Discussion

The expanding knowledge of the impact of the gut microbiota on cognition and brain function has led to a number of studies investigating potential avenues to manipulate the microbiota-gut-brain connection, including the use of probiotics, prebiotics or dietary fibre. While some benefits of prebiotic supplementation on host physiology have previously been illustrated in animals (Savignac et al., 2013; Mika et al., 2014) and humans (Hull et al., 2012; Holscher, 2017), evidence for the impact on mental health and cognition is limited. Thus, in this study we aimed to investigate the potential of a prebiotic fibre, PDX, on cognitive function, acute stress response and inflammation. Overall, PDX only had limited effects on the outcome measures assessed in this cohort. Contrary to our hypothesis, diversity of the gut microbiota did not change after PDX supplementation and only an increase in *Ruminiclostridium* 5 was detected. While a modest improvement in cognitive flexibility and sustained attention of participants as well as attenuation of the increase in adhesion receptor CD62L on classical monocytes after the acute stressor was observed, PDX supplementation neither impacted the other measures of the inflammatory profile and stress responses nor mood or psychological outcomes.

In animal models, combined with other pre- or probiotics, PDX affected cognitive function such as exploratory behaviour, recognition memory and neurochemistry (Fleming et al., 2019) and could attenuate the effects of early life stress on anxiety-like behaviour and learning (McVey Neufeld et al., 2019). Here, a subtle improvement in cognitive flexibility as measured by the IED task and in sustained attention as shown by the increased number of total correct rejections in the RVP task after PDX supplementation was observed. Cognitive flexibility is an executive function that allows to switch from thinking about one concept to another or to alternate between tasks (Kim et al., 2011). The IED task specifically tests behavioural set shifting abilities involving visual discrimination and attentional set formation maintenance, shifting and flexibility of attention that depend on fronto-striatal areas. The RVP task, on the other hand, measures sustained attention and speed of processing. Sustained

attention is the ability to maintain attention through a set of stimuli over a long period of time which is mainly regulated by the right prefrontal and superior parietal cortex (Pardo et al., 1991). The present data indicating better performance in cognitive flexibility and sustained attention after PDX supplementation aligns with observations from other studies showing improved performance in these two cognitive processes after prebiotic consumption. For example, in the same attentional set shift task adapted for rodents as the one used in this study, rats consuming B-GOS required fewer trials shifting from an intra- to an extra-dimensional set (Gronier et al., 2018). In human studies, B-GOS supplementation improved sustained attention measured as vigilance or tonic alertness in healthy volunteers (Schmidt et al., 2015) and enhanced cognitive performance, specifically executive functioning, in patients with psychosis (Kao et al., 2019). Taken together, results from the cognitive assessment in this study could provide evidence for a psychobiotic and cognitive-enhancing effect of PDX supplementation.

Contrary to previous studies showing that PDX increases microbial diversity and supports the growth of beneficial bacteria (such as increases in *Faecalibacterium*, *Akkermansia* and *Dialister* (Roytjo and Ouwehand, 2014)), in this cohort no statistically significant changes in microbiota diversity and only minimal changes in composition were observed. However, it should be noted that the effect of PDX on the microbial composition varies greatly from study to study (Roytjo and Ouwehand, 2014), mainly due to different dosages of PDX (8g (Costabile et al., 2012) vs. 21g (Holscher et al., 2015; Holscher, 2017)), differences in microbiota analysis (next generation sequencing (Holscher et al., 2015) vs. denaturing gradient gel electrophoresis (Costabile et al., 2012)) and difference in population characteristics (healthy vs. obese, age, gender etc.). Additionally, PDX has been shown to only impact outcome measures of weight management, gut barrier function and inflammation in combination with other probiotic supplementations (Stenman et al., 2016) or elicited microbial changes in obese populations, in which an aberration in microbiota composition is commonly observed (Hibberd et al., 2019). It is also increasingly being established that the magnitude of response to prebiotic

supplementation and dietary interventions depends on factors such as baseline microbiota composition or dietary habits. Thereby, baseline microbial diversity, richness and prior stability could be predictors of the responsiveness of a participant to an intervention (Salonen et al., 2014; Tap et al., 2015). The lack of vast microbial changes observed herein could therefore be explained by the relatively low dose of the prebiotic fibre, an unresponsive baseline microbial community or the healthy status of the population.

Interestingly, we found a significant increase in *Ruminiclostridium 5* abundance after PDX supplementation. Little is known about the function of *Ruminiclostridium 5* in humans. Lower levels of *Ruminiclostridium 5* were found in patients with kidney stones (Tang et al., 2018) and in a rat model of acute necrotizing pancreatitis (Chen et al., 2017), potentially indicating an important role of this bacterium in inflammatory diseases. Additional studies are needed to decipher the potential function of *Ruminiclostridium 5* in human physiology. It is worth mentioning that dietary intake over the study period did not change majorly, so that the increase in *Ruminiclostridium 5* could mainly be attributed to the PDX.

Monocytes have previously been described as a pathway for the microbiota-brain communication and levels of the murine counterpart of classical monocytes have been shown to change in response to acute stress (van de Wouw et al., 2019; van de Wouw et al., 2020). Previous research indicated that PDX could have immune modulatory effects (Schley and Field, 2002), such as increased secretion of IgA and decreased expression of cyclooxygenase-2 in rats and pigs (Peuranen et al., 2004; Fava et al., 2007). Here, we report that the increase in CD62L receptor expression on classical monocytes that was observed after the acute stressor during the placebo phase was attenuated by PDX supplementation. CD62L receptor expression can be used as a marker of acute stress-responsiveness, as it has been reported to be changed in response to acute stress in rats (Dhabhar et al., 2012). Based on the preliminary data presented herein, it could be hypothesized that potential anti-

inflammatory effects of PDX may mediate the immune-to-brain communication. In animal models, classical monocytes have been implicated in microbiota-associated changes in cognition (Mohle et al., 2016). In obese individuals, low grade inflammation was reported as a mediator between obesity and performance in the IED task (Lasselin et al., 2016). These studies suggest that PDX supplementation controls the inflammatory response and might have potential health implications for patients with chronic inflammatory diseases, potentially including brain health.

There are several limitations to this study. First, the sample size is relatively small. Although we acknowledge the exploratory nature of this study and the implications of a small sample size for uncertainty regarding type I and specifically type II error, the cross over design reduces confounding variables and provides statistical control for between-participant variation, thus allowing for a lower sample size to achieve similar power as non-crossover design studies. Nevertheless, we acknowledge that the current findings should be interpreted with caution due to the preliminary nature and should be used to inform larger clinical trials. Additionally, while a 4-week washout period and carryover design was applied to this study, the potential of carryover effects cannot be ruled out. The lack of effects of PDX on psychopathology, anxiety or stress could be partially explained by the healthy study cohort, showing low baseline depression, perceived stress or anxiety levels for which it would be difficult to observe significant changes. On the other hand, cognitive function in a healthy population might have more scope for improvement; however, larger studies are needed to investigate the specific benefits of PDX on cognition. The limited amount of changes observed in the microbiota composition could be due to the limitations of 16S rRNA sequencing. More detailed taxonomic classification using shot-gun metagenomic sequencing might be required to fully understand the impact of PDX on the microbiota composition and function. Nevertheless, while some studies observed more pronounced changes in the microbial profile after PDX supplementation, others report very subtle changes in the microbiome composition similar to the results observed herein (Roytio and Ouwehand, 2014). Lastly, although maltodextrin is frequently used as the placebo in various studies, it can readily be

digested and thus might have psychological and physiological effects (Kendig et al., 2014), potentially impacting some outcomes measures of this study.

Despite these limitations, to the best of our knowledge the data generated from this study is the first human study investigating the impact of PDX on brain health, thereby generating preliminary evidence to inform future clinical trials.. Previously, mechanisms by which PDX can exert beneficial effects on the host have been suggested to include microbial metabolites affecting hormonal concentrations in the blood (Tolhurst et al., 2012). Although the data provided from this study is insufficient to draw conclusions on underlying mechanisms, it could be hypothesized that the prebiotic PDX improved cognitive function through reducing inflammatory status, potentially mediated by *Ruminiclostridium 5* abundance. However, future larger studies using cognition as the primary outcome are warranted to fully understand whether PDX could be a potential psychobiotic candidate to support cognitive function and unravel underlying mechanisms.

6.2 Bifidobacterium longum Counters the Effects of Obesity: Partial Successful Translation from Rodent to Human

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6.2.1 Abstract

6.2.1.1 Background

The human gut microbiota has emerged as a key factor in the development of obesity. Certain probiotic strains have shown anti-obesity effects. The objective of this study was to investigate whether *Bifidobacterium longum* APC1472 has anti-obesity effects in high-fat diet (HFD)-induced obese mice and whether *B. longum* APC1472 supplementation reduces body-mass index (BMI) in healthy overweight/obese individuals as the primary outcome. *B. longum* APC1472 effects on waist-to-hip ratio (W/H ratio) and on obesity-associated plasma biomarkers were analysed as secondary outcomes.

6.2.1.2 Methods

B. longum APC1472 was administered to HFD-fed C57BL/6 mice in drinking water for 16 weeks. In the human intervention trial, participants received *B. longum* APC1472 or placebo supplementation for 12 weeks, during which primary and secondary outcomes were measured at the beginning and end of the intervention.

6.2.1.3 Findings

B. longum APC1472 supplementation was associated with decreased bodyweight, fat depots accumulation and increased glucose tolerance in HFD-fed mice. While, in healthy overweight/obese adults, the supplementation of *B. longum* APC1472 strain did not change primary outcomes of BMI (0.03, 95% CI [-0.4, 0.3]) or W/H ratio (0.003, 95% CI [-0.01, 0.01]), a positive effect on the secondary outcome of fasting blood glucose levels was found (-0.299, 95% CI [-0.44, -0.09]).

6.2.1.4 Interpretation

This study shows a positive translational effect of *B. longum* APC1472 on fasting blood glucose from a preclinical mouse model of obesity to a human intervention study in otherwise healthy overweight and obese individuals. This highlights the promising potential of *B. longum* APC1472 to be developed as a valuable supplement in reducing specific markers of obesity.

6.2.2 Introduction

Obesity is one of the most pervasive, chronic diseases globally, in both developed and developing countries, contributing to at least 2.8 million deaths annually and significantly impacting the healthcare system (WHO, 2018). The growing obesity epidemic is associated with increases in several comorbidities, such as cardiovascular disease, stroke, metabolic syndrome, type 2 diabetes and cancer (Tchernof and Despres, 2013; Narayanaswami and Dvoskin, 2017). Current available anti-obesity therapeutics are limited and associated with poor efficacy and adverse side effects (Bloom et al., 2008; Torres-Fuentes et al., 2014). Diet and exercise have been demonstrated to be the most potent in reducing obesity symptomatology (Fock and Khoo, 2013). In addition, natural compounds and their derivatives have been proposed as safer anti-obesity alternatives, either as functional foods or nutraceuticals (Torres-Fuentes et al., 2014).

The gut microbiota has emerged as a key component in the development of obesity and modulates the host's physiology and metabolism, including energy harvest, storage and expenditure (DiBaise et al., 2012; Torres-Fuentes et al., 2014; Rosenbaum et al., 2015; Patterson et al., 2016; Maruvada et al., 2017; Torres-Fuentes et al., 2017; van de Wouw et al., 2017; Cani et al., 2019). Preclinical and clinical evidence demonstrating the critical role of the gastrointestinal microbiota on host metabolism is steadily increasing. For example, germ-free mice are protected against obesity and are significantly leaner than normal control mice despite consuming more calories (Backhed et al., 2004). In addition, faecal transplantation from obese donors was shown to replicate the obese phenotype in lean germ-free mice independent of diet (Ley et al., 2005; Ley et al., 2006; Ridaura et al., 2013). Moreover, accelerated post-dieting weight regain is associated with a persistent intestinal microbiome signature after successful dieting in obese mice (Thaiss et al., 2016).

However, the exact mechanisms of how diet-induced changes in gut microbiota affect gut-brain signalling, including host metabolism, appetite regulation and brain health, are currently still lacking (Adan et al., 2019; Ezra-Nevo et al., 2020). Interestingly, the obese-associated microbiota has been shown to have an increased

capability to harvest energy from food and contributes to host insulin resistance, gut permeability, low-grade inflammation, and fat deposition (Cani and Delzenne, 2009; Khan et al., 2016). Intestinal microbiota-derived metabolites have also been shown to impact the central regulation of appetite (Fetissov, 2017; Sandhu et al., 2017; Torres-Fuentes et al., 2017). For example, certain bacterial strains modify gut peptides secretion, such as glucagon-like peptide (GLP)-1, thus contributing to hypothalamic appetite and satiety signalling via afferent nerve fibres of the vagus nerve as well as by direct secretion into the circulatory system (Everard and Cani, 2014; Sandhu et al., 2017). Furthermore, germ-free mice display marked decreases in expression of intestinal satiety peptides, including cholecystokinin (CCK), peptide tyrosine-tyrosine (PYY) and GLP-1 and also lower circulating levels of leptin and ghrelin (Duca et al., 2012). In addition, serum ghrelin levels are negatively correlated with the abundance of certain microbiota, including *Bifidobacterium* and *Lactobacillus* species (Queipo-Ortuno et al., 2013). And intake of the prebiotic oligofructose, which promotes the growth of *Bifidobacterium* and *Lactobacillus*, decreases the secretion of ghrelin in obese humans (Parnell and Reimer, 2009). Taken together, modulation of the gut microbiota is emerging as a promising strategy for the management of obesity and obesity-related disorders such as type-2 diabetes and cardiovascular disease (DiBaise et al., 2012; Torres-Fuentes et al., 2014; Patterson et al., 2016; Torres-Fuentes et al., 2017; Peng et al., 2018).

Several probiotic strains with different anti-obesity effects in humans have been identified (Jung et al., 2013; Rajkumar et al., 2014; Torres-Fuentes et al., 2014; Zarrati et al., 2014; Stenman et al., 2016; Sabico et al., 2017; Kim et al., 2018; Hibberd et al., 2019). The bacterial strain *B. longum* APC1472 has recently been shown to modulate ghrelinergic signalling *in vitro* (Torres-Fuentes et al., 2019), highlighting the therapeutic potential for host metabolism, appetite and obesity modulation. The ghrelin receptor (GHS-R1a) is activated by the endogenous hormone, ghrelin, the first and only known peripheral orexigenic peptide, which regulates peripheral metabolism and energy expenditure as well as centrally regulated homeostatic appetite and food-motivated reward signalling, governing eating behavior and food intake (Kojima et al., 1999; Tschop et al., 2000; Cummings et al., 2001; Nakazato et

al., 2001; Muller et al., 2015). Interestingly, obese individuals have attenuated postprandial suppression of ghrelin and a blunted nocturnal plasma ghrelin increase, reinforcing aberrant ghrelinergic signalling in obesity (Yildiz et al., 2004; le Roux et al., 2005). While the precise site of action of ghrelin is somewhat controversial (Abizaid, 2009; Abizaid and Horvath, 2012; Howick et al., 2017), the high prevalence of the ghrelin receptor throughout the small and large intestine, make it a likely target for interaction with the gut microbiota and thus may hold potential as a local therapeutic target (Takeshita et al., 2006).

As such, we investigated *B. longum* APC1472 for its ability to ameliorate high-fat diet (HFD)-induced obesity in mice, and significant effects on adiposity and metabolism were observed. Based on these promising effects of *B. longum* in the preclinical model, we subsequently investigated whether it could improve obesity symptomatology in healthy overweight/obese adults. The primary objective of the human intervention study was to determine whether a 12-week daily supplementation of *B. longum* APC1472 decreases body-mass index (BMI), while the secondary objective was to investigate the effects on waist-to-hip ratio (W/H ratio), and biomarkers associated with obesity, such as glucose, insulin, HbA1c and ghrelin levels. The exploratory objectives were to investigate the impact of *B. longum* APC1472 on the gut microbiota composition and diversity, peripheral inflammatory profile, stress hormone profile, self-reported stress, anxiety and satiety.

6.2.3 Methods

6.2.3.1 Animal study

6.2.3.1.1 Animals, diets and ethical approval

Five-week-old male C57BL/6 mice (Harlan Laboratories, UK) (40 mice, n=8-10 per group) were housed in groups of 2 mice per cage in standard holding cages with free access to food and water in the animal care facility of University College Cork. The holding room temperature ($21 \pm 1^\circ\text{C}$) and humidity ($55 \pm 10\%$) were controlled under a 12 h light/dark cycle (lights on 7.00 AM, lights off 7.00 PM). The mice were fed a low-fat diet (LFD) (10% fat (kcal/100 g), D12450B, Research Diet, USA) or a high-fat diet (HFD) (45% fat (kcal/100 g), D12451, Research Diet, USA) for 16 weeks. Food intake was recorded once per week and calculated on the basis of two mice per cage and five cages per group. The data were reported as cumulative food intake per mouse. Bodyweight was monitored weekly for 15 weeks. Experiments were conducted in accordance with the European Directive 86/609/EEC and the Recommendation 2007/526/65/EC and were approved by the Animal Experimentation Ethics Committee of University College Cork.

6.2.3.1.2 *In vivo* probiotic administration

Bifidobacterium longum APC1472 was grown anaerobically in De Man, Rogosa and Sharpe (MRS) medium as previously described (Torres-Fuentes et al., 2019). The bacterial cell pellet was washed and concentrated in sterile phosphate buffered saline (PBS) containing 25% Glycerol (v/v) to an end concentration of $\sim 7.5 \times 10^9$ CFU/mL, aliquoted and stored at -80°C . Aliquots were defrosted daily just prior to the start of the dark phase and diluted to $\sim 2 \times 10^8$ CFU/mL in drinking water for administration to LFD-fed and HFD-fed mice for 16 weeks. Water intake was monitored throughout the experiment. Drinking water containing an equivalent end concentration of sterile PBS (2% v/v) and glycerol (0.5% v/v) was administered to control mice. Water was replaced for probiotic/vehicle-free water every morning. *B. longum* APC1472 survival in drinking water (distilled water) in ambient temperature and oxygen content was tested over 24 h prior to the start of the experiment. Bacteria counts (CFU/mL) did not decrease over 1 log unit for the first 12 hours

suggesting adequate viability of the strain upon the time of consumption (**Figure S1A**). No significant changes in water intake were observed within the same diet groups (**Figure S1B**).

6.2.3.1.3 *In vivo* Glucose tolerance test

Glucose tolerance was assessed after 15 weeks of treatment as previously described (Suez et al., 2014), with minor modifications. Briefly, mice were fasted for 7 hours during the light phase, with free access to water. Glucose levels were measured in tail vein blood using a glucometer (Bayer, UK) immediately before and 15, 30, 60, 90 and 120 min after intraperitoneal injection of glucose (1 g/kg of body weight in sterile saline).

6.2.3.1.4 Murine tissue sampling

Mice were euthanized by decapitation. Trunk blood was collected in tubes containing 25 μ M dipeptidyl peptidase IV (DPP-IV) inhibitor, 2x protease inhibitor cocktail (Roche) (diluted in PBS) and 0.1% Na₂ EDTA for an expected blood volume of 400 μ L, centrifuged at 3500 g for 15 min at 4°C and placed on dry ice until storage at –80°C for further analysis. Adipose depots (epididymal, subcutaneous, mesenteric and retroperitoneal) were dissected and weighed. Whole-brains were collected and placed for 8-10 sec into ice-cold isopentane. All tissues were frozen on dry ice and subsequently stored at –80°C for further analysis.

6.2.3.1.5 Murine biochemical analysis

Plasma insulin and leptin levels were analysed by ELISA using the MILLIPLEX® MAP Mouse Metabolic Hormone Magnetic Bead Panel (Millipore, MMHMAG-44K) accordingly to the manufacturer's instructions. Plasma ghrelin levels were analysed using the Rat/Mouse Ghrelin (Total) ELISA Kits (Millipore, EZGRA-88K). Triglycerides levels were analysed with a Triglyceride Quantification Kit (Abcam Ltd, ab65336) following the to manufacturer's instructions. Corticosterone levels were assayed using ELISA kits (Enzo Life Sciences, ADI-900-097) according to the manufacturer's instructions.

6.2.3.1.6 Murine RNA Isolation and Quantitative Real-Time PCR

Hypothalamus was dissected with a forceps (macropunch) from the frozen brain on dry ice and immediately processed for RNA extraction. Hypothalamus and epididymal adipose tissue total RNA were extracted using the mirVana™ miRNA Isolation kit (Ambion/Life Technologies, AM1560) and RNeasy® Lipid Tissue Mini Kit (Qiagen, 74804), respectively with DNase treatment using Turbo DNA-free (Ambion/Life Technologies, AM1907) according to the manufacturer's recommendations. Equal amounts of RNA were first reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). Real-time PCR was performed using TaqMan Universal Master Mix II, no Uracil-N glycosylase (UNG) on a LightCycler®480 System (Roche). Mouse β -actin control mix Probe dye: VIC-MGB (Applied Biosystems, 4352341E) was used as an endogenous control. Target genes were amplified with probes designed by Integrated DNA Technologies (Table S1). Cycle threshold (Ct) values were recorded, normalized to its endogenous control and transformed to relative gene expression value using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Each sample was analysed in triplicate for both target gene and endogenous control. The gene expression levels for each animal was calculated considering the mean from each of these triplicates.

6.2.3.2 Human intervention study

6.2.3.2.1 Human intervention study outline

This study has a parallel-controlled design. In total, 150 individuals were screened, after which 124 were randomized into the treatment groups (Placebo: n = 50; Treatment: n = 74). The aim of the first visit of the participant was to assess participants for their eligibility to participate in the study and explain which procedures would be undertaken. Subjects were given an appointment for the next visit within a 3-week period. At the second visit, all baseline data and biologics were recorded, which was also done after 6 (visit 3) and 12 weeks (visit 4) of placebo or *B. longum* APC1472 treatment. Vital signs, anthropometric measurements and medical history were recorded. For women of childbearing age, a urine sample was collected for a pregnancy test. Fasting blood samples (20 mL) were collected to assess glucose, insulin, HbA1c, lipid profiles, satiety/appetite hormone profiles, and inflammatory

profiles. Saliva samples were collected for the assessment of the cortisol awakening response, as well as a stool sample for the microbiota analysis and short-chain fatty acid (SCFA) quantification. Questionnaires were administered to assess self-reported stress, anxiety, hunger/satiety, exercise and diet.

Participants were asked to take one capsule per day, providing a daily dose of 1×10^{10} CFU. Subjects, study facilitators, nurses and research analysts were kept blind as to in which group they belonged. The randomisation of treatment schedules was carried out by a computer-generated program. The remaining study product was collected to check for compliance following visits 3 and 4 (Haynes et al., 1980).

6.2.3.2.2 Inclusion and exclusion criteria

The inclusion criteria were as follows: subjects had to give written informed consent; had to be between 18-65 years of age; had a BMI between 28-34.9; had a W/H ratio ≥ 0.88 for males and ≥ 0.83 for females; had to be willing to consume the investigational product daily for the duration of the study. Subjects were excluded if they were pregnant, lactating, or female and wish to become a parent during the study; regularly took probiotics; were hypersensitive to any of the components of the test product; were severely immune-compromised (i.e. HIV positive, transplant patient, antirejection medications, on a steroid for >30 days, or underwent chemotherapy or radiotherapy within the last year); had Type 1 or Type 2 Diabetes Mellitus; had a history of bariatric surgery; had taken anti-obesity medication in the previous 12-weeks; were actively, or has within the last 3 months, participating in a weight loss program or incurred a weight change of more than 3 kg during the past 3 months; had a life-threatening illness; was on Metformin, anti-psychotic drugs or any medication that the investigator determined could impact the results of the study; had commenced use of anti-hypertensive drugs, anti-depressive drugs, statins or any other medication that the investigator determined could impact the results of the study within 3-months of randomisation date; had a history of co-existing gastrointestinal, and/or gynaecological, and/or urologic pathology (e.g. colon cancer, colitis, Crohn's Disease, Celiac, Endometriosis, prostate cancer) or lactose intolerance; had a history of drug and/or alcohol abuse; was currently, or planning, to participate in another study during the study period; had a history of non-

compliance; had been on antibiotics in the 12-weeks prior to randomisation; or consumed vitamin D supplements (>5000 IU/d). 17.3% of all screened participants were excluded due to these exclusion criteria.

Subjects were removed from the study if they independently elected to withdraw; he/she developed any condition which contravened the original criteria; or was considered at any point to be unsuitable to continue the study, at the discretion of the investigator.

6.2.3.2.3 Study setting and ethical approval

The study was conducted in accordance with the ethical principles set forth in the current version of the Declaration of Helsinki (seventh version, October 2013), the International Conference on Harmonization E6 (R2) Good Clinical Practice (ICH GCP, November 2016) and all applicable local regulatory requirements (i.e. Clinical Research Ethics Committee of the Cork Teaching Hospitals). This study was registered with ClinicalTrials.gov (NCT04042181). The CONSORT diagram of this study is depicted in **Figure 6.2-1**, the study layout is depicted in **Figure S2**. This study was run by Atlantia Food Clinical Trials (Cork, Ireland) (study reference: AFRCO-088).

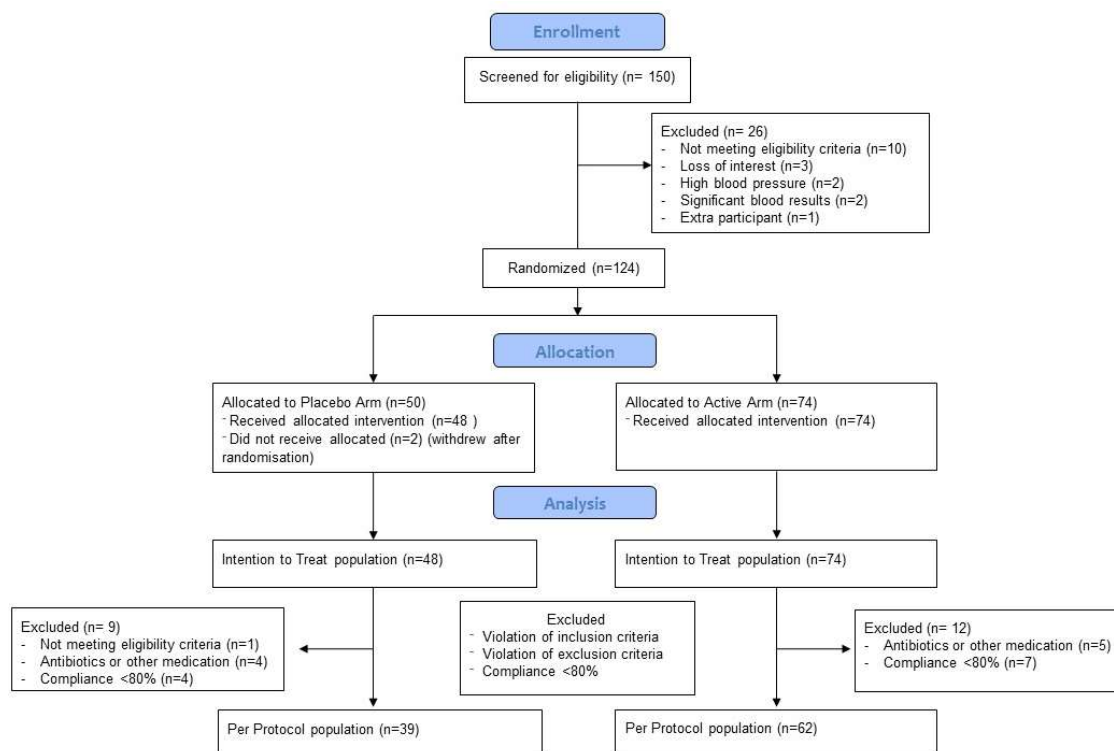


Figure 7.21. Consort diagram. Number of healthy overweight/obese participants that were assessed for eligibility and excluded or allocated to the trial, treated, followed, and analysed.

6.2.3.2.4 Randomisation and blinding

The investigational product arrived on site labelled with randomisation number. A randomisation list was generated by an independent statistician. Participants were assigned a randomisation number in chronological order from this randomisation list. The study team, participants and researchers were unaware which randomisation numbers were active or placebo. Blinding was undone after all data had been analyzed.

6.2.3.2.5 Study recruitment

Subjects were recruited through the database of Atlantia Food Clinical Research Trials, general practitioners' offices and by posting adverts in local newspapers. Subjects underwent an initial phone screen. Eligible subjects were scheduled for a screening visit. Subjects received €300 upon completion of the study to cover costs and expenses incurred.

6.2.3.2.6 Product formulation and dosage

Bifidobacterium longum has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA). *B. longum* APC1472 grown culture and the corresponding placebo were freeze-dried (Sacco SRI, Italy) and provided as hydroxypropylmethylcellulose (HPMC) capsules in PE bottles (Nutralinea, Italy). The freeze-dried powder of the strain was blended with standard food-grade excipients to achieve the target dose of 1×10^{10} CFU. The excipients consisted of corn starch, magnesium stearate and silicon dioxide. The probiotic formulation consisted of *B. longum* APC1472, whereas the placebo contained maltodextrin. The product was stored at -20°C until distributed to the study participant and the participant was instructed to keep the product refrigerated. Participants returned any leftover product at their next visit, and the excess product was counted to check for compliance.

6.2.3.2.7 Collection and analysis of blood samples

Fasting blood samples were taken into EDTA tubes, fasting defined as refraining from food overnight (at least 10 hours), however drinking water was allowed throughout the duration of the fast. Samples for the analysis of active ghrelin were immediately treated with AEBSF (final concentration 1 mg/mL, Sigma, A8456), centrifuged and the resulting plasma was treated with HCl (final concentration 0.05N). Blood samples for the analysis using the U-PLEX assays were treated with DPP-IV inhibitor (final concentration 1%, Sigma, DPP4) and centrifuged. Blood plasma samples for other analyses did not undergo any additional processing, except for centrifugation. Centrifugation was performed at 1000g for 10 minutes at 4 °C, after which samples were aliquoted and either processed or stored at -80 °C for future analysis.

Blood plasma from visit 1 was used to measure urea, creatinine, bilirubin, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, gamma-glutamyl transferase, total protein, albumin, globulin, calcium, magnesium, phosphate, uric acid, cholesterol, HDL cholesterol, LDL cholesterol, total triglycerides, glucose, full blood count + 5-part diff. Safety blood, haematology and biochemistry parameters were analysed by Biomnis-Eurofins Ireland.

Blood from visits 2, 3 and 4 was used to measure total cholesterol, LDL, HDL, triglycerides HbA1c, glucose and insulin by Biomnis-Eurofins Ireland. Furthermore, blood plasma was assessed for active ghrelin levels using an ELISA (EMD Millipore, EZGRA-88BK) which was performed according to the manufacturer's instructions. Plates were read at 405 nm with a correction at 590 nm using the synergy HT plate reader (Biotek instruments). Blood plasma was also assessed for metabolic and inflammatory biomarkers using custom U-PLEX assays (MSD, K151ACM-2), which were also performed according to the manufacturer's instructions. Blood plasma samples were diluted 1:3 for the U-PLEX assays. U-PLEX markers were linked as following; Plate 1: 1) Leptin, 2) PYY, 3) GLP-1 – total, 4) IFN γ , 5) IL-4, 7) TNF- α , 8) IL-10, 9) C-peptide, 10) Ghrelin – total; Plate 2: 1) GLP-1 – active. The working solution was supplemented with DPP-IV inhibitor (final concentration 1%, Sigma, DPP4). Plates were read using the MESO QuickPlex SQ 120. Duplicates with $\geq 20\%$ coefficient of variability were re-analysed. Samples did not undergo any additional freeze-thaw cycles.

6.2.3.2.8 Collection and analysis of cortisol awakening response samples

To monitor the cortisol awakening response, saliva from visits 2 and 4 was collected in Salivette devices (Sarstedt, 51.1534.500) immediately upon awakening, and after 30, 45 and 60 minutes. Participants were instructed to keep samples in the fridge until delivery at the visit time, after which they were centrifuged at 1500 g for 5 min, the saliva was harvested and immediately stored at -80 °C. Salivary cortisol concentrations were quantified using ELISA kits (Enzo life sciences, ADI-901-071), which were performed according to the manufacturer's instructions. Saliva samples were diluted 1:2. Plates were read at 405 nm with a correction at 580 nm using the synergy HT plate reader (Biotek instruments). Duplicates with $\geq 20\%$ coefficient of variability were re-analysed. Samples did not undergo any additional freeze-thaw cycles. Cortisol awakening response was calculated using the area under the curve increase (AUCi). Briefly, data from the 30-, 45- and 60-minute time-points were normalized (delta) to the samples taken immediately upon awakening, after which the sum was taken of the 30-, 45- and 60-minute time-points (Stalder et al., 2016).

6.2.3.3 Murine and Human Microbiota

6.2.3.3.1 Murine and Human Microbiota sequencing

Murine cecal DNA was isolated using the QIAamp Fast DNA Stool Mini kit (Qiagen) as previously described and kept at -20°C until further analysis (Burokas et al., 2017). Isolated DNA was quantified on a NanoDrop ND2000 spectrophotometer (Thermo Scientific, DE) and used for 16S ribosomal RNA sequencing by Illumina MiSeq System (Illumina Inc., USA) according to the manufacturer's instructions. Briefly, PCR amplicons (primers for V3-V4 hypervariable region of the 16S rRNA gene: F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGCCTACGGGNGGCWGCAG-3') and R (5'-GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACH VGGGTATCTAATCC-3') were purified and libraries prepared as previously described (Burokas et al., 2017). Briefly, the 16S V3-V4 amplicons were generated using Kapa HiFi HS ready mix and purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). The Nextera XT Index Kit (Illumina Inc., USA) was used to barcode each sample. PCR products were cleaned using AMPure XP beads and a magnetic 96-well plate. Final barcoded amplicons were measured using the Qubit dsDNA High Sensitivity assay kit on the Qubit 3.0 fluorometer, diluted to 5 ng/μL and pooled. The PCR products from both PCR steps (Amplicon & Indexing) were visualised in agarose gels stained with SYBR Safe DNA gel stain (Invitrogen). Samples were sequenced at Clinical-Microbiomics, Denmark on the Illumina MiSeq platform using a 2 x 300 bp kit. After sequencing, reads were assembled, processed and analysed as previously described (Burokas et al., 2017). In the microbiota composition analysis, LDA Effect Size (LEfSe: Linear Discriminant Analysis Effect Size) was used as an algorithm with default settings on the interface Galaxy (<http://huttenhower.sph.harvard.edu/lefse/>) (Segata et al., 2011) to identify taxa with differentiating abundances. The differentially abundant features are ranked by effect size after undergoing linear discriminant analysis (LDA), using an effect size threshold of 2 (log₁₀ scale). In non-technical terms, LEfSe pre-selects features that are different between groups and then tries to fit a model to see how well these features explain the groups. The score is an average between the effect size and how well the model fits, after which they are transformed to a value between -6 and 6.

Principal coordinates Analysis (PCoA) was performed based on Bray-Curtis beta diversity distances using the Adonis function in the “vegan” (2.4-3) package for R (version 3.3.1).

For the human intervention study, faecal sample collection and DNA extraction was performed as previously described (see supplementary material for details) (Yu and Morrison, 2004). The DNA samples were processed according to the Illumina 16S Metagenomic Sequencing Library Preparation instructions as described above for the murine DNA samples. Final barcoded amplicons were measured using the Qubit dsDNA High Sensitivity assay kit on the Qubit 3.0 fluorometer, diluted to 8.3 ng/μL, pooled and sent for sequencing. Microbiome analysis was carried out in R (version 3.6.1) with Rstudio (version 1.2.1335). DADA2 was used to denoise and call amplicon sequence variants (ASVs). Taxonomy was assigned using the SILVA SSUREf database version 132. ASVs unknown on a genus level were excluded, as well as ASVs present in two or fewer samples. The ALDEx2 library used to compute the centered log-ratio transformed values of the remaining taxa (Fernandes et al., 2014). For principal components analysis (PCA), a pairwise implementation of the `adonis()` PERMANOVA function in the `vegan` library followed by the Bonferroni-Holm correction was used to test for difference in β -diversity in terms of Aitchison distance (source: Oksanen, Jari, et al. "Package 'vegan'." Community ecology package, version 2.9 (2013): 1-295). Differential abundance was assessed using a pairwise implementation of the `aldex.test()` function, followed by Benjamini-Hochberg correction. In all cases, a q -value < 0.1 was considered significant. α -diversity was computed using the `iNEXT` library (Hsieh and Chao, 2017).

6.2.3.4 Faecal SCFA quantification

Faecal samples were homogenised with acidified water (HCl pH 3) at a ratio of 1:7.5 w/v and analyzed by gas chromatography flame ionisation detection (GC-FID) using a Varian 3800 GC system, fitted with an Agilent DB-FFAP column (30 mL x 0.32mm ID x 0.25 μm df; Agilent) and a flame ionisation detector with a CP-8400 auto-sampler. Helium was employed as the carrier gas at an initial flow rate of 1.3 mL/min. The initial oven temperature was 50 °C, was maintained for 30 seconds, raised to 140°C at 10 °C/min and held for 30 seconds, before being increased to 240°C at 20 °C/min,

and held for 5 minutes (total run time 20 minutes). The temperatures of the detector and the injection port were 300 °C and 240 °C, respectively. A split-less injection of 0.2 µL was carried out for each sample or standard using a 10 µL syringe (Agilent) installed to a CP-8400 auto-sampler (Varian). A 5 m guard column was installed between the injector and analytical column (Restek). Peak integration was performed using Varian Star Chromatography Workstation version 6.0 software. Vials containing 1800 µL of water were run between each sample duplicates as blanks to control for any potential carryover. Standards were included in each run to maintain the calibration. For further details on sample and standards preparation see supplementary information.

6.2.3.5 Questionnaires

Using self-report scales, participants were assessed for perceived stress using Cohen's Perceived Stress Scale and anxiety and depression using the Hospital Anxiety and Depression Scale (HADS) at baseline, after 6 and after 12 weeks, as previously described (Cohen et al., 1983; Zigmond and Snaith, 1983). In addition, satiety/hunger was determined using a visual analogue Hunger/Satiety scale, physical activity using the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003). Nutrient intake was assessed using a Food Frequency Questionnaire (FFQ), as previously described (Harrington et al., 2011).

6.2.3.6 Statistical analysis

Preclinical data were assessed for normality using the Shapiro-Wilk test. Normally distributed data were analysed using a two-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc test. Non-parametric datasets were analysed using the Kruskal-Wallis test, followed by the Mann-Whitney U test with Bonferroni adjustment of p-values. Body weight changes and glucose levels in glucose tolerance test were analysed with a two way repeated-measures ANOVA (with Diet and Probiotic as two independent factors and Time as a repeated-measured factor), followed by LSD post hoc test at each time point. Statistical analysis was performed using SPSS software (IBM SPSS statistics 22). Preclinical data are represented as mean \pm SEM.

For the human intervention study, differences between the treatment and placebo groups at the last visit (i.e. visit 4) were analysed using an analysis of covariance (ANCOVA), correcting for baseline variance (i.e. visit 2) and sex. Comparisons between baseline measurements (visit 2) and post-intervention measurements (visit 4) were analysed using an unpaired student's T-test. Analyses were performed on the intention to treat populations. Statistical analysis was performed using SPSS software version 26 (IBM Corp). Data in table are presented as mean \pm SEM or 95% CI. P-Values <0.05 were considered statistically significant. Partial eta-squared (η^2) was used to estimate effect size (Tabachnick and Fidell, 2012). Effect sizes were interpreted as following: $\eta^2 \leq 0.06$ was considered small, $0.06 > \eta^2 \leq 0.14$ was considered moderate, $\eta^2 \geq 0.14$ was considered large.

6.2.4 Results

6.2.4.1 *B. longum* APC1472 decreases body weight gain and fat depots accumulation in obese mice

B. longum APC1472 decreased body weight gain after 15 weeks of administration ($F(1, 33) = 4.751, p = 0.037$) (**Figure 6.1-2A, 2B**). HFD feeding increased caloric intake ($F(1, 15) = 9.229, p = 0.008$) (**Figure S1C**), body weight ($F(1, 33) = 29.715, p < 0.001$) (**Figure 6.2-2A**) and fat depot accumulation (mesenteric ($F(1, 33) = 61.328, p < 0.001$), retroperitoneal ($F(1, 32) = 128.409, p < 0.001$), subcutaneous ($F(1, 31) = 124.091, p < 0.001$) and epididymal ($F(1, 33) = 81.673, p < 0.001$)) (**Figure 6.2-2C, D, E, F**). Pairwise comparisons showed a significant decreased body weight effect of *B. longum* APC1472 in HFD-fed mice ($p = 0.047$) (**Figure 6.2-2B**), which was independent of caloric intake (**Figure S1**). Furthermore, the administration of *B. longum* APC1472 significantly reduced fat depot accumulation (mesenteric ($F(1, 33) = 5.908, p = 0.021$), and subcutaneous ($F(1, 33) = 4.270, p = 0.047$)) (**Figure 6.2-2C, D, E, F**). Finally, pairwise comparisons revealed a significant decreased fat depot accumulation effect of *B. longum* APC1472 administration in HFD-fed mice (mesenteric $p = 0.002$, retroperitoneal $p = 0.05$ and subcutaneous $p = 0.023$).

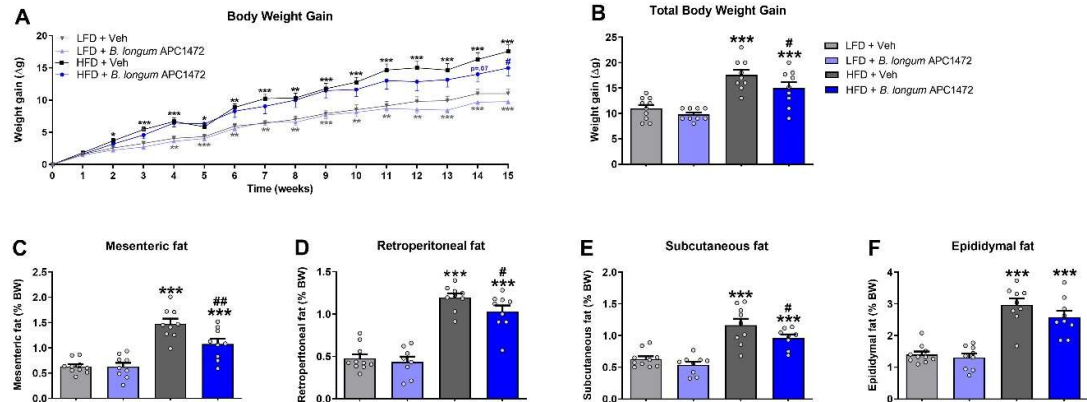


Figure 6.2-2. Effects of *Bifidobacterium longum* APC1472 on body weight and fat depots accumulation in mice. (A) Weekly body weight gain, (B) total body weight gain and (C) mesenteric, (D) retroperitoneal, (E) subcutaneous and (F) epididymal fat depots accumulation (% of total body weight) in control mice treated with drinking water containing sterile PBS (2% v/v) and glycerol (0.5% v/v) and fed a control low-fat diet (LFD) ($n=10$) or a high-fat diet (HFD) ($n=9$) and in mice treated with *B. longum* APC1472 in drinking water (2×10^8 CFU/mL) and fed a LFD ($n=9$ in A, B, C, E and F; $n=8$ in D) or a HFD ($n=9$ in A, B, C, D, and F; $n=8$ in E) for 15 (A, and B) or 16 weeks (C, D, E and F). Data are shown as mean \pm SEM. Data are significant different ($p < 0.05$) accordingly to Repeated Measures ANOVA (A) or two-way ANOVA followed by LSD post-hoc test (B, C, D, E and F). * indicates significant diet treatment effect (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and # indicates significant *B. longum* APC1472 treatment effect (# $p < 0.05$, ## $p < 0.01$).

6.2.4.2 *B. longum* APC1472 administration improves glucose tolerance, circulating levels of leptin and corticosterone in obese mice

Effect of HFD feeding ($F(5, 155) = 3.321$, $p = 0.007$) and a *B. longum* APC1472 supplementation ($F(5, 155) = 4.792$, $p < 0.001$) effect were observed, as well as an interaction effect between these two factors and time ($F(5, 155) = 3.307$, $p = 0.007$) in the glucose tolerance test. Supplementation with *B. longum* APC1472 normalized glucose levels after 15 mins of glucose administration in HFD-fed obese mice ($p = 0.006$) and also significantly decreased glucose after 90 ($p = 0.019$) and 120 min ($p = 0.018$) respectively (**Figure 6.2-3A**) as determined by 2 way ANOVAs at each individual timepoint. Moreover, HFD feeding ($F(1, 33) = 29.761$, $p < 0.001$), *B. longum* APC1472 ($F(1, 33) = 4.425$, $p = 0.043$) and interaction effects between these two factors ($F(1, 33) = 5.337$, $p = 0.027$) were also observed when analyzing the area under the glucose levels curve (AUC) (**Figure 6.2-3B**), with *B. longum* APC1472 administration significantly reducing AUC in HFD-fed mice ($p = 0.003$) as determined by post-hoc comparison (**Figure 6.2-3B**). In addition, both a HFD feeding ($F(1, 33) = 9.167$, $p = 0.005$) and a *B. longum* APC1472 effect ($F(1, 33) = 4.796$, $p = 0.036$) were observed for non-fasting insulin levels (**Figure 6.2-3C**). Interestingly, *B. longum*

APC1472 reduced non-fasting insulin levels in LFD-fed mice ($p = 0.054$) but not in HFD-fed mice (**Figure 6.2-3C**). However, for fasting glucose levels, only a HFD feeding effect was observed ($F(1, 32) = 29.153, p < 0.001$) (**Figure 6.2-3D**). Moreover, both a HFD feeding ($F(1, 31) = 30.926, p < 0.001$) and a *B. longum* APC1472 effect ($F(1, 31) = 17.917, p < 0.001$) was observed for epididymal insulin receptor substrate 1 (*IRS-1*) expression (**Figure 6.2-3E**). Post-hoc comparisons determined that interestingly, *B. longum* APC1472 significantly reduced *IRS-1* expression in both LFD ($p = 0.002$) and HFD-fed mice ($p = 0.011$) (**Figure 6.2-3E**). Both a HFD feeding ($F(1, 33) = 38.023, p < 0.001$) and a *B. longum* APC1472 ($F(1, 33) = 5.340, p = 0.027$) effect as well as an interaction effect ($F(1, 33) = 4.237, p = 0.048$) was observed for fasting leptin levels (**Figure 6.2-3F**). The effect of HFD on leptin levels was attenuated by *B. longum* APC1472 treatment ($p = 0.004$). Finally, we found a significant *B. longum* APC1472 treatment effect ($F(1, 32) = 7.774, p = 0.009$) for plasma corticosterone levels (**Figure 6.2-3G**). Administration of *B. longum* APC1472 significantly decreased plasma corticosterone levels in HFD-fed mice ($p = 0.011$) (**Figure 6.2-3G**), which may have contributed to its overall impact on glucose homeostasis.

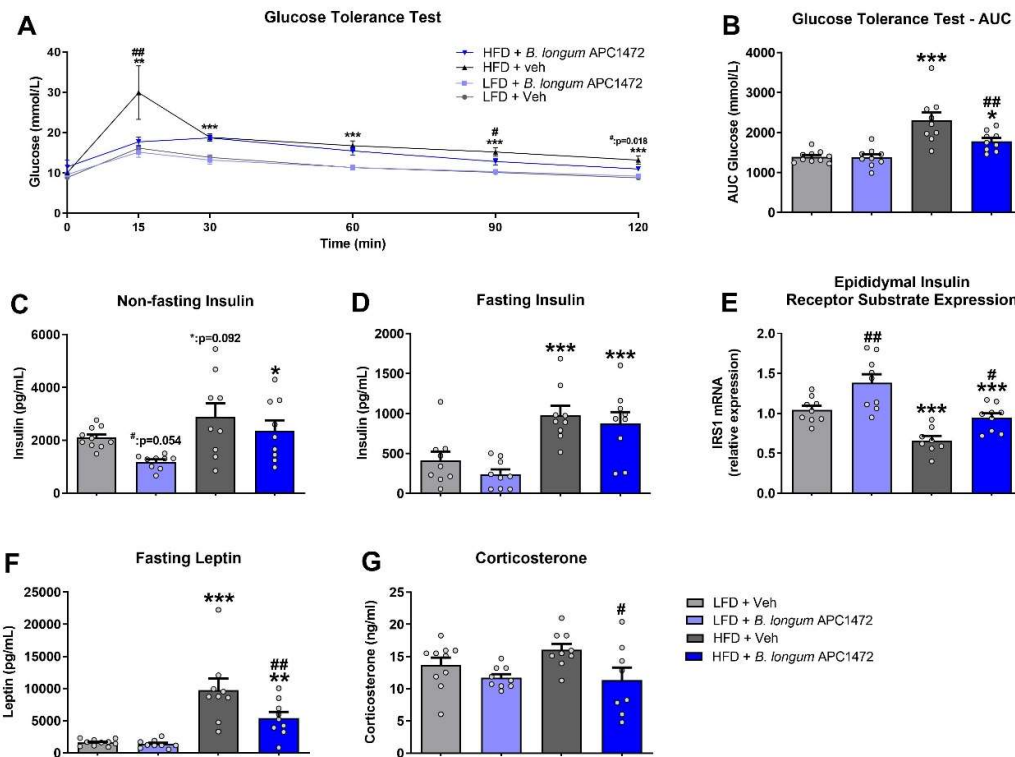


Figure 6.2-3. *Bifidobacterium longum* APC1472 improved glucose tolerance, leptin plasma levels and stress-induced corticosterone circulating levels in high-fat diet-induced obesity in mice. (A and B) Glucose tolerance test (GTT) glucose curve and area under the curve (AUC) after 1 g/kg glucose challenge, (C and D) non-fasting and fasting insulin plasma levels, (E) fasting leptin plasma levels, (F) epididymal fat insulin receptor substrate (IRS)-1 mRNA expression and (G) fasting-induced corticosterone plasma in control mice treated with drinking water containing sterile PBS (2% v/v) and glycerol (0.5% v/v) and fed a control low-fat diet (LFD) ($n=10$ in A, B, C, E, F and G) or a high-fat diet (HFD) ($n=9$ in A, B, C, D, E and G; $n=8$ in F) and in mice treated with *B. longum* APC1472 in drinking water (2×10^8 CFU/mL) and fed a LFD ($n=9$ in A, B, C, D, E and F; $n=8$ in G) or a HFD ($n=9$ in A, B, C, D, E and F; $n=8$ in G) for 15 (A, B, C) or 16 weeks (D, E, F and G). Data are shown as mean \pm SEM. Data are significant different ($p < 0.05$) according to Repeated Measures ANOVA (A) or two-way ANOVA followed by LSD post-hoc test (B, C, D, E, F and G). * indicates significant diet treatment effect (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and # indicates significant *B. longum* APC1472 treatment effect (# $p < 0.05$, ## $p < 0.01$).

6.2.4.3 B. *longum* APC1472 induces changes of hypothalamic neuropeptide expression in mice

Analysis of the gene expression levels of hypothalamic neuropeptides involved in appetite modulation revealed a significant HFD effect on the gene expression of the orexigenic peptide agouti-related protein (*AgRP*) ($F(1, 33) = 10.412$, $p = 0.003$) but a non-significant reduction in neuropeptide Y (*NPY*) expression (Figure S3). Interestingly, both a *B. longum* APC1472 effect ($F(1, 33) = 7.820$, $p = 0.009$) and an interaction effect ($F(1, 33) = 5.881$, $p = 0.021$) were observed for cocaine- and

amphetamine-regulated transcript (*CART*) expression (**Figure S3**). Indeed, *B. longum* APC1472 administration significantly reduced *CART* expression in HFD-fed mice ($p = 0.001$). While a reduced expression was observed for the anorexigenic pro-opiomelanocortin (*POMC*) gene expression in HFD-fed animals treated with *B. longum* APC1472 compared to HFD-fed, this did not reach statistical significance. Finally, no significant change in leptin (*LEP-R*) nor ghrelin (*GHS-R1a*) receptor expression was observed (**Figure S3**).

6.2.4.4 Human Intervention Study population

In the human study, no significant differences were observed in weight, BMI, W/H ratio, age, height, sex, ethnicity, mode of delivery, alcohol consumption, medical/surgical history and compliance at baseline between *B. longum* APC1472 treatment and placebo groups (**Table 1**). We did observe an increased prevalence of concomitant medical or nutritional supplement consumption in the treatment group (48.6%) compared to the placebo group (33.3%). In addition, we also observed differences in the socioeconomic profile where there was a lower prevalence of employers and managers in the treatment group (2/74) compared to the placebo group (4/48). Similarly, we observed a lower prevalence of past smokers in the treatment group (28/74) compared to the placebo group (9/48). In conclusion, the baseline characteristics of our placebo group and *B. longum* APC1472 group are mostly the same.

Physical activity and food intake patterns were also assessed, using self-report questionnaires, throughout the study (**Table S2, S3**). No differences in physical activity levels or calorie, macro- and micronutrient intake were observed over the 12-week treatment period or between the placebo and *B. longum* APC1472 group.

6.2.4.4.1 Adverse Events

There were seven adverse events (6 placebo participants and 1 treatment participant) that were possibly related to the investigational product. The adverse event of the treatment participant was constipation. The remaining 6 adverse events were; gastro-intestinal discomfort and increased appetite; bloating; increased

flatulence; aches in joints and increased temperature; rash on knees, elbows, scalp and red blotches on chest & upper arm.

Table 1. Baseline characteristics of subjects in the placebo and treatment arms at visit 1 (screening visit). Abbreviations: BMI = Body-mass index; W/H ratio = waist-to-hip ratio.

Variable	Placebo (n = 48, mean ± STD)	B. longum APC1472 (n = 74, mean ± STD)
Weight (kg)	87.9 ± 1.7	89.0 ± 1.3
BMI	31.2 ± 0.3	30.8 ± 0.2
W/H ratio	0.95 ± 0.01	0.96 ± 0.01
Age (years)	46.3 ± 9.9	44.9 ± 11.4
Height (m)	1.67 ± 0.10	1.70 ± 0.09
Sex (no. of subject (%))		
Male	19 (39.6 %)	34 (45.9 %)
Female	29 (60.4 %)	40 (54.1 %)
Race or ethnicity (no. of subject (%))		
Caucasian	48 (100 %)	73 (98.6 %)
Arabic	0 (0 %)	1 (1.4 %)
Socioeconomic status (no. of subject (%))		
Non-manual	15 (31.3 %)	21 (28.4 %)
Lower Professional	14 (29.2 %)	19 (25.7 %)
Manual skilled	4 (8.3 %)	8 (10.8 %)
Semi-skilled	4 (8.3 %)	8 (10.8 %)
Employers and managers	4 (8.3 %)	2 (2.7 %)
Own account workers	3 (6.3 %)	7 (9.5 %)
Higher Professional	3 (6.3 %)	5 (6.8 %)
All others gainfully occupied and unknown	1 (2.1 %)	2 (2.7 %)
Farmer	0 (0 %)	1 (1.4 %)
Unskilled	0 (0 %)	1 (1.4 %)
Smoking status (no. of subject (%))		
Non-smoker	22 (45.8 %)	40 (54.1 %)
Past smoker	17 (35.4 %)	28 (37.8 %)
Current smoker	9 (18.8 %)	6 (8.1 %)

Alcohol consumption (mean \pm SEM)		
Units per week	4.97 \pm 0.68	4.31 \pm 0.46
Currently on concomitant medical or nutritional supplements (no. of subject (%))		
Yes	16 (33.3 %)	36 (48.6 %)
No	32 (66.7 %)	38 (51.4 %)
Compliance (% product consumed)		
Week 6	95.8 \pm 1.2	97.9 \pm 0.8
Week 12	94.0 \pm 2.0	97.2 \pm 1.2

6.2.4.5 *B. longum* APC1472 does not affect BMI and W/H ratio in humans

The primary outcome of this study was to investigate whether *B. longum* APC1472 supplementation could alter BMI, and a secondary outcome of change in W/H ratio was included to support the primary outcome. However, no differences were observed in BMI and W/H ratio over the 12-week treatment period, or between the placebo and *B. longum* APC1472 treatment groups (Figure 6.2-4).

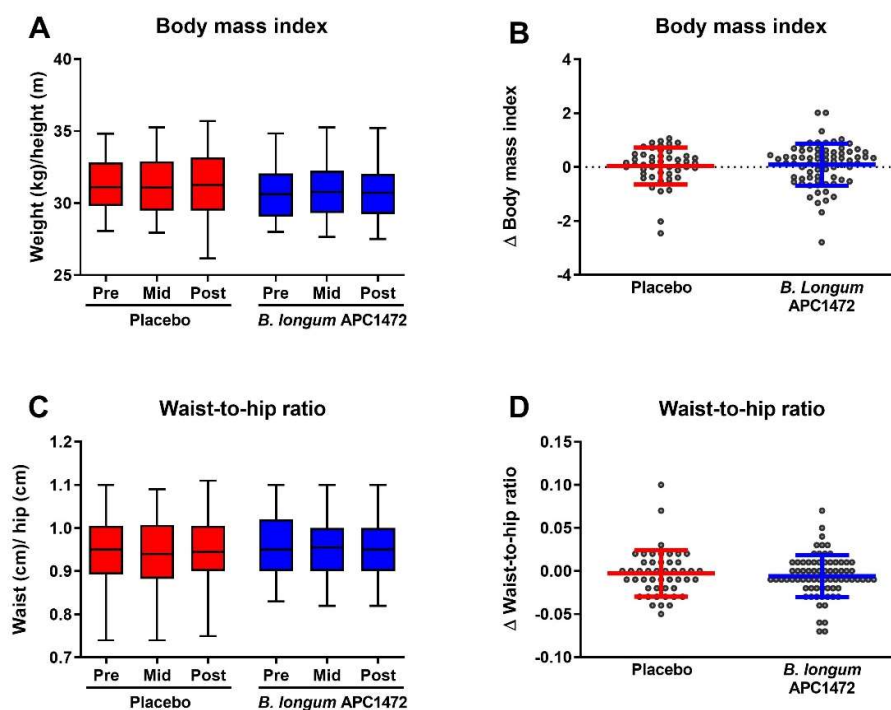


Figure 6.2-4. *B. longum* APC1472 supplementation does not impact BMI and W/H ratio in overweight and obese individuals. Body mass index (BMI) (A, B) and waist-to-hip ratio (W/H ratio) (C, D) were measured as the beginning of the study (pre), after 6 weeks (mid) and after 12 weeks (post) of treatment. All BMI and W/H ratio data are depicted of all 3 timepoints (A, C), as well as the change after 12 weeks compared to at the beginning of the study (B, D). Data are depicted as boxplot or scatter dot plot, where the dots depict individual datapoints. $N = 48$ for the placebo group and $n = 74$ for the *B. longum* APC1472 treatment group.

6.2.4.6 *B. longum* APC1472 improves fasting glucose levels independent of other blood markers of energy metabolism and satiety in humans

We subsequently measured markers associated with host energy metabolism and satiety as part of the secondary and exploratory outcome measures (**Figure 6.2-5** and **Table S4** for full statistical results). Here we observed that both the *B. longum* APC1472 and the placebo arm reduced fasting glucose levels over the 12-week treatment period (**Figure 6.2-5A**). However, glucose levels were 0.266 mmol/L (95% CI [-0.44, -0.09]) lower in the *B. longum* APC1472 group compared with the placebo group ($F(1,112) = 9.073$, $p = 0.003$) (**Figure 6.2-5B**). The effect size of the *B. longum* APC1472-induced decrease was moderate ($\eta^2 = 0.075$). We also observed that HbA1c levels decreased over the 12-week treatment period in both the placebo group ($t(62.372) = 4.277$, $p < 0.001$) and *B. longum* APC1472 treatment group ($t(85.983) = 5.787$, $p < 0.001$) (**Figure 6.2-5C**). However, there were no differences between the groups, indicating that the decrease in HbA1c levels is most likely explained by the 12-week treatment period or placebo effect. No changes were observed in other biomarkers of host metabolism such as insulin, C-peptide, ghrelin (active and total),

GLP-1 (active and total), PYY and leptin levels (Figure 6.2-5 E-T).

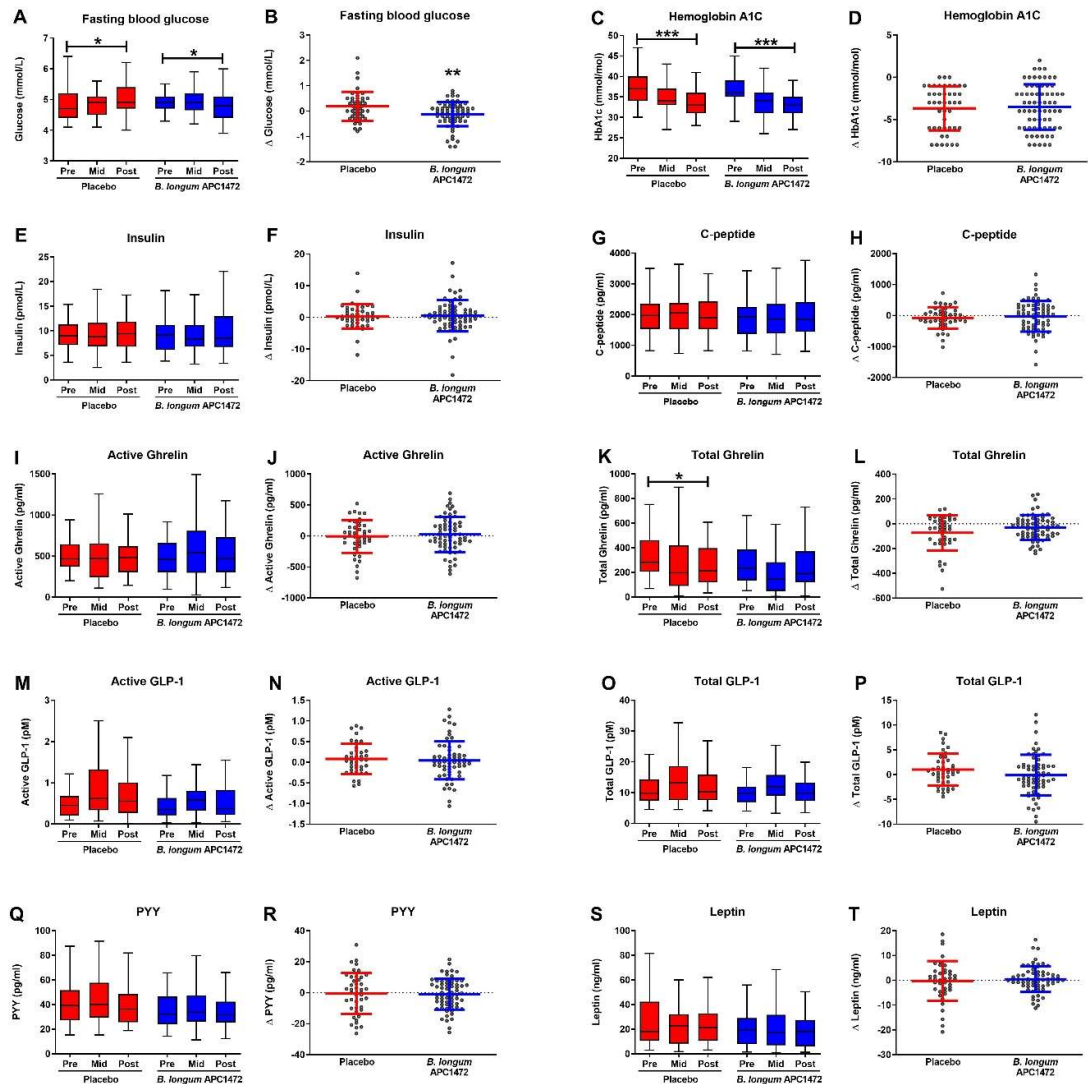


Figure 7.5-5. *B. longum* APC1472 supplementation reduces fasting blood glucose levels in overweight and obese individuals. Markers associated with host metabolism and satiety were measured as the beginning of the study (pre), after 6 weeks (mid) and after 12 weeks (post) of treatment. All data are depicted of all 3 timepoints (A, C, E, G, I, K, M, O, Q, R), as well as the change after 12 weeks compared to at the beginning of the study (B, D, F, H, J, L, N, P, R, T). Data are depicted as boxplot or scatter dot plot, where the dots depict individual datapoints. $N = 48$ for the placebo group and $n = 74$ for the *B. longum* APC1472 treatment group. * indicates a significant effect (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

6.2.4.7 *B. longum* APC1472 does not influence human lipid and inflammatory profiles in humans

It is well-known that obesity is associated with metabolic syndrome, hypertension and hyperlipidemia (Jarolimova et al., 2013). *B. longum* APC1472 did not impact lipid

profiles (i.e. cholesterol, triglycerides and LDL), and inflammatory profiles (i.e. IL-10, TNF- α and IFN γ) compared to the placebo group (**Table 2**). In addition, vital signs remained unaltered throughout the study (**Table S5**). These results reveal that *B. longum* APC1472 did not evoke any negative effects on vital signs or induced any inflammation. Interestingly, even though no significant changes were observed in HDL levels over the 12-week treatment period, a small increase in HDL levels was observed in the *B. longum* APC1472 group ($F(1,117) = 3.260$, $p = 0.074$). The effect-size of the increase in HDL levels was small ($\eta^2 = 0.027$).

6.2.4.8 *B. longum* APC1472 does not affect satiety, mood, perceived stress and cortisol awakening response in humans

Considering that the gut microbiota has been implicated in the modulation of host mood and food intake behaviour (van de Wouw et al., 2017; Lach et al., 2018), we investigated whether *B. longum* APC1472 could improve levels of the stress hormone cortisol upon waking (i.e. cortisol awakening response), or self-reported measures of satiety, and self-reported measures of mood (i.e. perceived stress, anxiety and depression) (**Table 3**). *B. longum* APC1472 did not impact cortisol awakening response, or self-reported satiety, perceived stress, anxiety and depression measures.

6.2.4.9 *B. longum* APC1472 improves fasting glucose levels, active ghrelin and cortisol awakening response in obese individuals

Participants in this study were either overweight ($n = 40$; $28 \leq \text{BMI} < 30$) or obese ($n = 82$; $30 \leq \text{BMI} < 35$). It is possible that *B. longum* APC1472 could evoke a stronger effect in obese individuals as they have a stronger phenotype compared to overweight individuals. As such, we investigated whether any of the anthropomorphic measures, blood biomarkers and measures of mood were affected by *B. longum* APC1472 in the obese subpopulation only compared to placebo (**Figure 6.2-6** and **Table S7-11** for population characteristics and full statistical results). Similar to the analysis on the entire study population, *B. longum* APC1472 and placebo reduced fasting glucose levels over the 12-week treatment period (**Figure 6.2-6A**). However, glucose levels were 0.295 mmol/L (95% CI [-0.5, -0.1]) lower in the *B. longum* APC1472 group compared to the placebo group ($F(1,75) = 7.566$, $p =$

0.007), with a moderate effect size ($\eta^2 = 0.092$) (**Figure 6.2-6B**). Furthermore, *B. longum* APC1472 increased active ghrelin levels ($F(1,74) = 4.903, p = 0.030$), with a moderate effect size ($\eta^2 = 0.062$). *B. longum* APC1472 also reduced cortisol awakening response ($F(1,51) = 4.415, p = 0.041$), with a moderate effect size ($\eta^2 = 0.080$).

Overall, these results show beneficial effects of *B. longum* APC1472 on fasting plasma glucose levels, active ghrelin levels and cortisol awakening response in obese individuals. It is also important to note that the effect size in the obese subpopulation ($\eta^2 = 0.092$) was bigger than the effect size in the overall study population ($\eta^2 = 0.075$). This indicates that *B. longum* APC1472 has a more robust beneficial effect on fasting glucose levels in obese, rather than in overweight, individuals.

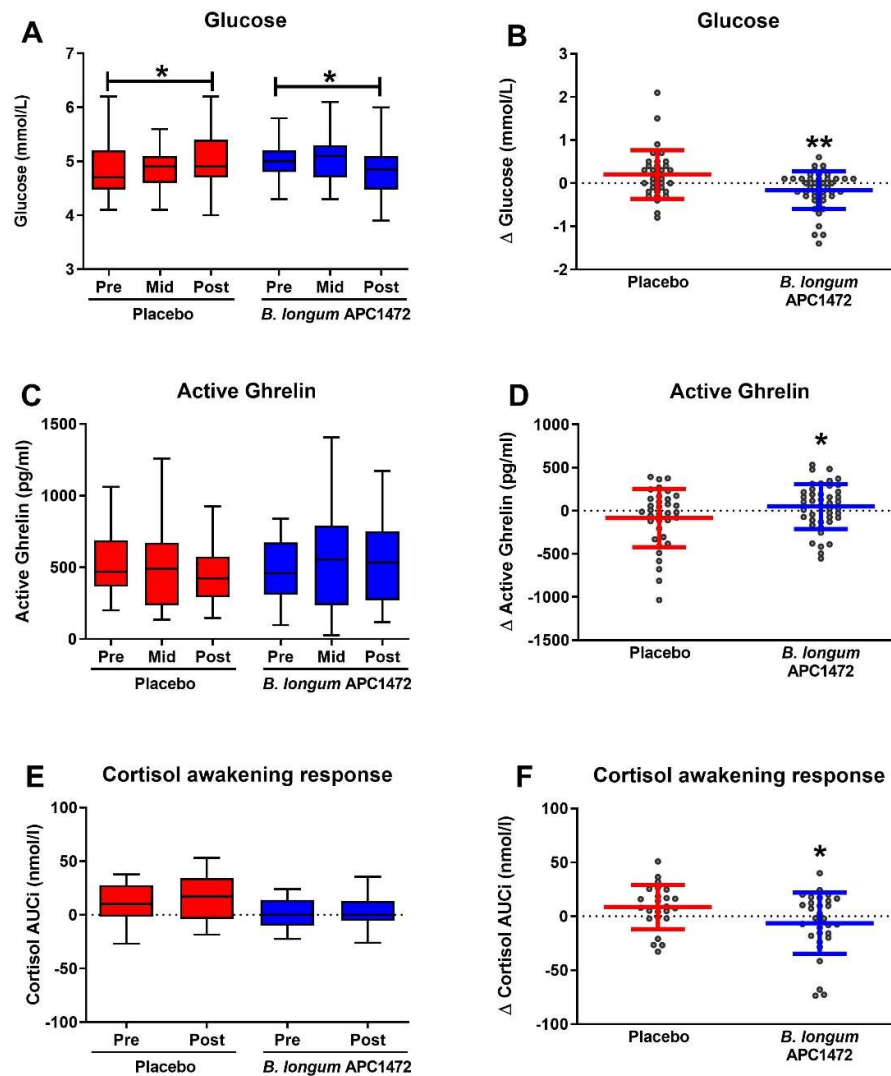


Figure 6.2-6. *B. longum* APC1472 supplementation reduces fasting blood glucose levels and cortisol awakening response and increase active ghrelin in obese individuals. Fasting glucose and active ghrelin levels were measured at the beginning of the study (pre), after 6 weeks (mid) and after 12 weeks (post) of treatment. Cortisol awakening response was only assessed at the beginning of the study. All data are depicted of all 3 timepoints (A, C, E), as well as the change after 12 weeks compared to at the beginning of the study (B, D, F). Data are depicted as boxplot or scatter dot plot, where the dots depict individual datapoints. $N = 36$ for the placebo group and $n = 46$ for the *B. longum* APC1472 treatment group. * indicates a significant effect (* $p < 0.05$, ** $p < 0.01$)

6.2.4.10 *B. longum* APC1472 does not induce major rearrangements on the microbiota composition but increases the abundance of *Bifidobacterium*

We subsequently investigated whether the observed changes induced by the *B. longum* APC1472 strain were mediated in part through modulation of the gut microbiota. Investigations into the cecal microbiota in the preclinical experiment revealed that there was a significant dissimilarity in beta diversity between LFD- and HFD-fed mice ($p < 0.01$) (**Figure S5A**), with a decreased relative abundances of Bacteroidetes phylum and increased relative abundances of Firmicutes class Clostridia, respectively (**Figure S5B**), which is in line with previous studies (Nadal et al., 2009; Clarke et al., 2012). Different phylotypes were responsible for the cecal microbiota differences among the treatment groups (**Figure S5C**), showing increments on different Firmicutes members in HFD-fed mice treated with *B. longum* APC1472. Moreover, *B. longum* APC1472 partially ameliorated the HFD-induced decrease in Bifidobacteriaceae relative abundance ($p = 0.054$, adjusted $p = 0.170$) (**Figure S5D**).

Analysis of the faecal microbiota in the human intervention study revealed that *B. longum* APC1472 did not impact the alpha diversity indices (Shannon, Simpson and Chao1, **Figure 6.2-7A-C**). Furthermore, the overall composition of the microbiota remained unaffected as determined by the PCA analysis of the beta diversity (**Figure 6.2-7D**). *B. longum* APC1472 did increase *Bifidobacterium* relative abundance over the 12-week intervention period ($t(57) = -2.891$, $p = 0.005$), which was not observed in the placebo group (**Figure 6.2-7E**). This resulted in a higher *Bifidobacterium* abundance in the treatment group compared to the placebo group post-intervention ($F(3, 89) = 5.922$, $p = 0.017$) (**Figure 6.2-7F**). Similar results were observed in the obese subpopulation (**Figure S6**).

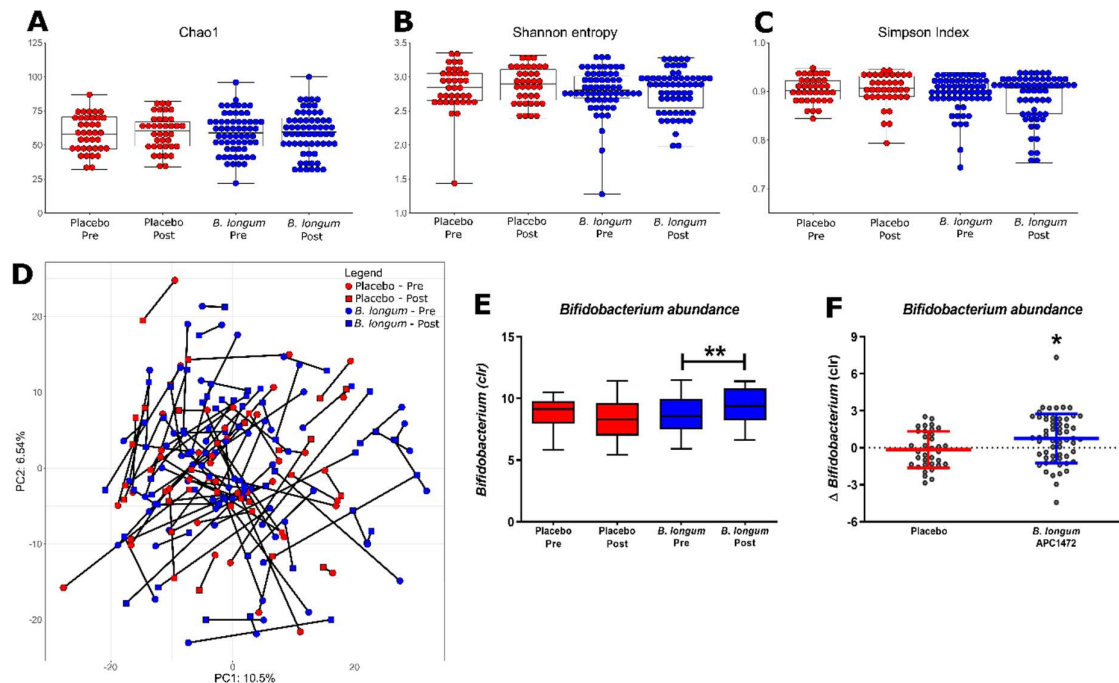


Figure 6.2-7. *B. longum* APC1472 increases *Bifidobacterium* abundance without impacting the overall composition of the gut microbiota in humans. The gut microbiota was assessed at the beginning (pre) and end of the study (12 weeks, post). Alpha (A-C) and beta diversity (D) were investigated, as well as the bacterial genera present (E-F). Microbial taxa were centre-log-transformed (CLR). Significant differences between pre and post were analysed using the Mann-Whitney U test, whereas treatment differences were analysed using an ANCOVA controlling for sex and pre-intervention *Bifidobacterium* abundance. Data are depicted as boxplot or scatter dot plot, where the dots depict individual datapoints. $N = 48$ for the placebo group and $n = 74$ for the *B. longum* APC1472 treatment group. * indicates a significant effect (* $p < 0.05$, ** $p < 0.01$).

Short-chain fatty acids (SCFAs) are potentially one of the most investigated gut microbiota-derived metabolites implicated in host energy metabolism and obesity symptomatology (Torres-Fuentes et al., 2017; van de Wouw et al., 2017). Analysis of faecal SCFA levels in human samples revealed no differences in levels of acetate, propionate, butyrate and valerate (Table S6). Furthermore, isobutyrate and isovalerate levels remained unaffected (Table S6).

6.2.5 Discussion

There has been an increased emphasis on gut microbiota-targeted therapeutics for the amelioration of obesity (Torres-Fuentes et al., 2014; Cani et al., 2019; Muscogiuri et al., 2019; Sivamaruthi et al., 2019). For example, recent studies have identified several probiotic strains with different anti-obesity effects, including members of the genus *Bifidobacterium* (Jung et al., 2013; Rajkumar et al., 2014; Torres-Fuentes et al.,

2014; Zarrati et al., 2014; Stenman et al., 2016; Sabico et al., 2017; Kim et al., 2018; Hibberd et al., 2019), but the exact mechanisms of action are still lacking. In the present study, we demonstrate that a novel isolated *B. longum* APC1472 strain, which was previously shown to attenuate ghrelinergic signalling (Torres-Fuentes et al., 2019), reduces body weight gain, fat depot size, glucose tolerance and leptin levels in a preclinical mouse model of HFD-induced obesity. Furthermore, when the *B. longum* APC1472 strain was investigated in a human cohort of healthy overweight and obese individuals a reduced fasting blood glucose level was observed. Noteworthy, stratification and analysis of the obese human subpopulation revealed that *B. longum* APC1472 was able to normalize active ghrelin levels and the cortisol awakening response, which are both dysregulated in obesity (Tschop et al., 2001; Yildiz et al., 2004; Cummings, 2006; Wester et al., 2014; Zigman et al., 2016; Jackson et al., 2017). This highlights the translational value of this novel *Bifidobacterium longum* species, *B. longum* APC1472, from a preclinical mouse model to a human intervention study where this probiotic positively impacts markers of obesity, which may be linked to the ghrelinergic effects previously demonstrated (Torres-Fuentes et al., 2019). Specifically, we found that in the preclinical mouse model of obesity, the supplementation with *B. longum* APC1472 significantly reduced fat depots and body weight gain in HFD-fed mice independent of energy intake. Furthermore, *B. longum* APC1472 significantly reduced circulating leptin levels in HFD-fed mice, which is in line with the reduction in fat depot size as leptin is released into the bloodstream in proportion to body fat mass (Friedman and Halaas, 1998). Notably, circulating levels of leptin were increased in HFD-fed mice compared to LFD-fed mice with no alterations in leptin receptor hypothalamic expression, suggesting no alterations in leptin sensitivity, as has been previously reported in obesity (Cui et al., 2017). No changes were observed in the hypothalamic expression of the orexigenic peptides *NPY* and *AgRP* following *B. longum* APC1472 supplementation in mice. Both *NPY* and *AgRP* are orexigenic peptides that increase food intake when overexpressed or when administered centrally (Ilnytska and Argyropoulos, 2008; Zhang et al., 2014) and HFD-fed mice demonstrate, as expected, a decrease in both of these orexigenic peptides. In contrast, increased hypothalamic expression of anorexigenic peptides such as *POMC* and *CART* in response to a high-fat diet has been suggested as a natural

feedback mechanism in order to maintain energy balance and body weight homeostasis (Bergen et al., 1999; Tian et al., 2004). The *B. longum* APC1472 was able to normalize the increased hypothalamic expression of the anorexigenic peptide *CART* in HFD-fed mice, suggesting a lower degree of energy imbalance and, therefore, a potential reduced metabolic dysfunction compared to HFD-fed mice. Moreover, *CART* is regulated by leptin and its expression is positively correlated with leptin levels (Wortley et al., 2004). Therefore, the decreased leptin levels observed in the *B. longum* APC1472-HFD group also support the observed decreased *CART* expression. This highlights the potential of *B. longum* APC1472 to modulate hypothalamic gene expression involved in energy homeostasis and appetite regulation, which warrants further investigation.

In the human intervention study, no differences were observed in the primary outcome of BMI, even though the *B. longum* APC1472 supplementation was able to reduce body weight gain in HFD-induced obese mice. Similarly, no differences were observed in the supportive secondary outcome W/H ratio. This discrepancy might be explained by the fact that the majority of the human intervention cohort was non-diabetic, whereas the HFD-induced obese mice had a decreased glucose tolerance, implying that host glucose metabolism may have been the main factor driving the reduction in body weight gain in the obese mice. It must also be noted that the treatment duration of the preclinical study was longer and, therefore, a longer treatment period in the human intervention study, or a higher treatment dosage, could have resulted in more significant differences and bigger effect-sizes. The 12-week duration of the human study may have been too short of a time to see significant changes in BMI and W/H ration. Age has also been shown to affect body fat distribution and metabolism increasing both the risk and the severity of obesity development (Jura and Kozak, 2016). Therefore, some of the discrepancies and lack of translation between the mice study and humans could be explained by the relatively low age of the mice (adolescence to adulthood) versus the human cohort with an average age at midlife. A low age may facilitate a better response to changes in metabolic and physiologic responses and therefore a higher capacity to positively respond to therapeutic interventions. Moreover, the administration strategies were

differences between both studies. The mouse study followed a prevention strategy as *B. longum* APC1472 was administered before obesity was established, while in the human study the participants were already obese at the time of administration and, therefore, presented a more severe condition to ameliorate.

Most notably, the *B. longum* APC1472 supplementation significantly improved glucose tolerance in HFD-induced obese mice. Similarly, *B. longum* APC1472 decreased fasting blood glucose levels in overweight/obese individuals (-0.266 mmol/L compared to placebo). It is important to note that the participants in this study had average fasting blood glucose levels of 5.0 mmol/L, which is considered healthy and non-diabetic (n = 11 were prediabetic). Above 5.6 mmol/L is considered prediabetic, whereas above 6.9 is considered diabetic (Emerging Risk Factors et al., 2010; Chatterjee et al., 2017). The data indicate that *B. longum* APC1472 may have a bigger effect-size on fasting blood glucose levels in a prediabetic or diabetic population, which warrants further investigations. This is further reinforced by the obese subpopulation analysis of the obese individuals, rather than overweight and obese combined, which revealed a fasting blood glucose level (-0.295 mmol/L compared to placebo), which constitutes a bigger effect-size in fasting blood glucose levels ($\eta^2 = 0.092$ vs 0.075), indicating a more potent treatment efficacy in obese individuals. This warrants further investigation into the effect of *B. longum* APC1472 in a cohort of prediabetic or diabetic individuals.

The underlying mechanisms for the decreased fasting blood glucose levels may be associated with the changes in ghrelinergic signalling, as *B. longum* APC1472 was found to attenuate ghrelinergic signalling *in vitro* (Torres-Fuentes et al., 2019) and ghrelin has been shown to be involved in glucose homeostasis via inhibition of insulin secretion (Ahima, 2006). Moreover, insulin receptor substrate 1 (IRS-1) has been reported to play a key role in glucose homeostasis being involved in glucose transporter 4 (GLUT-4) mobilization (Dong et al., 2006; Wang et al., 2009). Low IRS-1 expression levels have been associated with glucose and insulin sensitivity impairments (Dong et al., 2006; Wang et al., 2009). Therefore, increased IRS-1 expression in epididymal fat tissue of *B. longum* APC1472 treated mice may have also influenced glucose homeostasis. Nevertheless, glucose metabolism is multifactorial

and other mechanisms are likely also affected following the supplementation of the *B. longum* APC1472. However, while the biggest effect-size was observed on plasma glucose levels in both the preclinical and human intervention studies, it is also possible that the other observed effects are secondary to the decrease in plasma glucose levels.

Notably, obesity is associated with decreased circulating levels of ghrelin (Tschop et al., 2001; Yildiz et al., 2004), which we also observed in the HFD-fed mice and the reason why the ghrelinergic system has been implicated as a promising therapeutic target to combat obesity (Schellekens et al., 2013; Howick et al., 2017). Indeed, the “hunger hormone” ghrelin was first described as a growth hormone secretagogue, but its key role in the regulation of appetite, food intake, adiposity and metabolism have directed the main therapeutic focus of ghrelin and its receptor towards obesity research with promising anti-obesity potential (Tschop et al., 2000; Wren et al., 2001; Horvath et al., 2003; Schellekens et al., 2012; Collden et al., 2017; Cui et al., 2017; Howick et al., 2017). Interestingly, *B. longum* APC1472 supplementation increased levels of active ghrelin, but not total ghrelin levels, in healthy obese individuals. The increase in active ghrelin may indicate an amelioration of the deficiencies in ghrelinergic signalling associated with obesity. It is also interesting to note that *B. longum* APC1472 was selected on its ability to modulate the ghrelinergic system *in vitro* (Torres-Fuentes et al., 2019). Future studies are warranted to investigate if administration of other bacterial strains and their metabolites, including SCFAs, which equally showed the ability to modulate ghrelin signaling *in vitro* (Torres-Fuentes et al., 2019), have similar effects in obese individuals.

Furthermore, our data reveal that *B. longum* APC1472 decreased fasting corticosterone levels in HFD-induced obese mice, indicating the downregulation of the hypothalamic-pituitary-adrenal (HPA) axis. In line with these results, *B. longum* APC1472 reduced cortisol awakening responses in obese individuals. Dysregulation of the HPA axis, which is colloquially seen as the “body’s stress system”, is a risk factor for obesity-related conditions such as cardiovascular disease, insulin resistance and type 2 diabetes (Incollingo Rodriguez et al., 2015). Hence, the stress hormone cortisol (corticosterone in rodents), which is central in the HPA axis, has been shown to

promote the accumulation of fat cells and weight gain (Incollingo Rodriguez et al., 2015) and to regulate the function of pancreatic α and β cells affecting glucagon and insulin secretion (Kuo et al., 2015). As such, even though no changes were observed in insulin, the changes in cortisol awakening responses could indicate that the HPA axis has contributed to the *B. longum* APC1472-induced decrease in fasting blood glucose. Furthermore, the HPA axis is also affected by ghrelin, indicating that the observed changes in ghrelin could have also contributed to the changes in cortisol (Bali and Jaggi, 2016).

Finally, we investigated the effects of *B. longum* APC1472 treatment on gut microbiota composition. Overall, *B. longum* APC1472 treatment did not have a major impact on microbiota composition other than the partial restoration of *Bifidobacterium* levels in HFD-fed mice. These findings are in line with the effects of *B. longum* APC1472 on healthy human overweight and obese individuals and with other investigations on obesity using different probiotics strains, where major rearrangements on microbiota composition were also not observed (Depommier et al., 2019; Everard et al., 2019).

Of note, while the modulation of ghrelin receptor signalling by *B. longum* APC1472 strain may have contributed to an improved metabolic profile, we cannot rule out other beneficial anti-obesity effects. As such, future studies are warranted further investigating the mechanisms and metabolites through which *B. longum* APC1472 modulates host glucose homeostasis, with a focus on the ghrelinergic system.

In conclusion, we have demonstrated positive anti-obesity effects of a novel *B. longum* APC1472 in HFD-induced obese mice and a partial translation of these positive effects of *B. longum* APC1472 supplementation in otherwise healthy overweight and obese individuals. In particular, we show the promising potential of *B. longum* APC1472 to be developed as a valuable supplement in reducing specific markers of obesity, possibly via the ghrelinergic system. Most notably, the decrease in fasting plasma glucose induced by *B. longum* APC1472 may have clinically significant health indications for prediabetic and type 2 diabetes mellitus populations in particular.

6.3 Recipe for a Healthy Gut: Intake of Unpasteurised Milk Is Associated with Increased Lactobacillus Abundance in the Human Gut Microbiome

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6.3.1 Abstract:

6.3.1.1 Introduction:

The gut microbiota plays a role in gut-brain communication and can influence psychological functioning. Diet is one of the major determinants of gut microbiota composition. The impact of unpasteurized dairy products on the microbiota is unknown. In this observational study, we investigated the effect of a dietary change involving intake of unpasteurized dairy on gut microbiome composition and psychological status in participants undertaking a residential 12-week cookery course on an organic farm.

6.3.1.2 Methods:

Twenty-four participants completed the study. The majority of food consumed during their stay originated from the organic farm itself and included unpasteurized milk and dairy products. At the beginning and end of the course, participants provided faecal samples and completed self-report questionnaires on a variety of parameters including mood, anxiety and sleep. Nutrient intake was monitored with a food frequency questionnaire. Gut microbiota analysis was performed with 16S rRNA gene sequencing. Additionally, faecal short chain fatty acids (SCFAs) were measured.

6.3.1.3 Results:

Relative abundance of the genus *Lactobacillus* increased significantly between pre- and post-course time points. This increase was associated with participants intake of unpasteurized milk and dairy products. An increase in the faecal SCFA, valerate was observed along with an increase in the functional richness of the microbiome profile, as determined by measuring the predictive neuroactive potential using a gut-brain module approach.

6.3.1.4 Conclusions:

While concerns in relation to safety need to be considered, intake of unpasteurized milk and dairy products appear to be associated with the growth of the probiotic bacterial genus, *Lactobacillus* in the human gut. More research is needed on the

effect of dietary changes on gut microbiome composition, in particular in relation to the promotion of bacterial genera such as *Lactobacillus*, which are recognized as being beneficial for a range of physical and mental health outcomes.

6.3.2 Introduction

A growing body of evidence over the past decade has demonstrated the importance of the gut microbiome in all aspects of physical and mental health. While it is still unclear what exactly constitutes a 'healthy' gut microbiome, certain bacterial groups have been strongly associated with better health outcomes. *Lactobacillus* is one of the foremost genera considered to have probiotic properties (Di Cerbo et al., 2016). A probiotic is defined as a live microorganism which, when administered in adequate amounts, confers a health benefit on the host (Hill et al., 2014). The word '*psychobiotic*' is an expansion of this term and describes an organism which has been proven to be beneficial in relation to psychological functioning (Dinan et al., 2013). There have been a wide variety of studies undertaken in recent years which have demonstrated the benefit of a *Lactobacillus* probiotic, both mono- and multi-strain, for improving a range of health outcomes including obesity (John et al., 2018), diabetes (Hsieh et al., 2018), liver disease (Wong et al., 2013), cardiovascular disease (DiRienzo, 2014), gastrointestinal conditions (Wilkins and Sequoia, 2017) and neuropsychiatric disorders such as depression, anxiety and autism (Butler et al., 2019).

A key present-day challenge involves identifying the most effective ways of maintaining a healthy gut microbiome and promoting the growth of probiotic bacteria. While commercial probiotic products are widely available, there are concerns in relation to regulation, quality control, efficacy and cost (Kolacek et al., 2017). Dietary intake is one of the main factors regulating gut microbiome composition and food-based interventions can be tailored to each individual to modify their bacterial profile (Johnson et al., 2019). While unravelling the diet-microbiome relationship is a formidable task given the many confounding factors, attempts to do so have been made over the past decade. Gut microbiome profile has

been shown to be distinctly different in those living in rural areas with a traditional diet in comparison to urban-based westernized populations (De Filippo et al., 2010; Yatsunen et al., 2012; Clemente et al., 2015). Even when one accounts for contributions of human genetic and geographical factors between populations, subsistence methods and diet significantly impact gut microbiota composition (Jha et al., 2018). It is hypothesized that a 'microbiota insufficiency syndrome' has resulted from modern lifestyle with its highly processed diets, overuse of antibiotics and increased sanitation and that the 'industrialized' microbiota may be a major contributing factor in the rise of many non-communicable chronic diseases in westernized societies (Sonnenburg and Sonnenburg, 2019). Even as one moves from looking at the early ancestral microbiota to more recent times, significant changes in lifestyle have continued until relatively recently. Ireland, as with many countries in the developed world, was a predominantly agrarian society up until the mid-late 20th century. In 1966, over 30% of the workforce were employed in agriculture with this figure estimated at less than 5% in 2016 (Office, 2016). Consumption of unpasteurized milk was a common part of the diet of those living on farms and epidemiological studies suggest that it may have played a protective role against the development of allergies and atopic diseases (Braun-Fahrlander and von Mutius, 2011).

Despite food safety concerns, the consumption of unpasteurized milk appears to be growing in popularity (Buzby et al., 2013; Fagnani et al., 2019). To our knowledge, there are no studies exploring the impact of unpasteurized milk intake on the gut microbiome. In this observational study, we investigated the effect of a dietary change involving the intake of unpasteurized milk on gut microbiota composition, metabolites and psychological status in 24 participants undertaking a residential, farm-based, 12-week cookery course. Our centre had previously published a study (Quigley et al., 2013) on the microbiota composition of unpasteurised milk taken from Irish cows which would thus be representative of the expected microbiota composition of the raw milk that would be consumed by participants in our study. Given the reported high proportion of viable probiotic bacteria such as *Lactobacillus* (and other lactic acid bacteria including *Lactococcus* and *Leuconostoc*), along with

the fact that *Lactobacillus* are considered intrinsically resistant to gastric acid (Tannock, 2004), we hypothesized that a dietary change involving raw milk consumption would alter the gut microbiome of participants with a potential differential increase in the relative abundance of these probiotic bacterial groups.

6.3.3 Results

6.3.3.1 Participant characteristics

A total of 62 participants who were completing the 12-week course between May-July 2018 were informed about the study. Twenty-eight participants volunteered and underwent screening. Two were excluded; one had a chronic gastrointestinal disorder and another had taken antibiotics in the previous month. Twenty-six participants were enrolled with 24 (13 females, 11 males) completing the study; 2 failed to provide faecal samples. Of note, subject metadata and faecal samples were collected within the first three days of the course and again at week 11. The final week of the course (week 12) involved several examinations for students and thus, the associated increased stress during this week may have had the potential to influence findings. Our study sample comprised 24 participants; 13 females and 11 males. Baseline characteristics of participants, including age, body mass index (BMI), smoking status, sleep quality and exercise levels are shown in Table 1.

Table 1: Baseline Characteristics of participants

	Pre-course	Post-course	P-value
Number of participants	24		
Female; n (%)	13 (54%)		
Mean age; n (range)	30.25 (18-59)		
Smoking status; n (%)	7 (29)		

BMI (kg/m)	24.87 (3.42)	25.33 (3.61)	0.1
Physical activity (as measured by IPAQ score)	4757.52 (4614.74)	3271.52 (7280.05)	0.32
Sleep quality (as measured by PSQI)	5.36 (2.87)	4.95 (2.91)	0.25
Bristol stool scale score	3.78 (1.085)	4.04 (0.706)	0.39
GI-Visual Analogue Scale; Satisfaction with bowel habit	38.37 (33.757)	27.29 (27.98)	0.25

(BMI: Body Mass Index, IPAQ: International Physical Activity Questionnaire; PSQI: Pittsburgh Sleep Quality Index)

6.3.3.2 Changes in diet

Based on food frequency questionnaire (FFQ) analysis (Tables 2 and 3), there was no change in total calorie intake during the course. In terms of macronutrient intake, protein and carbohydrate intake remained unchanged and though total fat consumption increased, this change did not reach statistical significance (mean increase (g) from 94 ± 35 to 128 ± 66 , $p=0.08$). With regards to micronutrients, Vitamin A (μg) intake increased significantly (715 ± 577 to 1505 ± 975 , $p=0.005$) as did Vitamin B12 (μg) (7.8 ± 3.6 to 11 ± 5.8 , $p=0.04$). Although intake of fruit reduced slightly (2.02 ± 1.2 to 1.38 ± 0.84 , $p=0.04$) consumption of vegetables and wholegrains did not change, nor did intake of alcohol or unhealthy foods such as sweets or snacks.

Table 2: Changes in dietary components between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Nutrient	Recommended daily intake*	Pre-course	Post-course	p-value
Kilocalories	2,000 – 2,400 (males; depending on activity level)	2264±1006	2723±1494	0.47
Protein (g)	10-35% of total energy	97±40 (17%)	109±57 (16%)	0.54
Fat (g)	20-35% of total calories	94±35 (37%)	128±66 (42%)	0.08
Carbohydrate (g)	45-65% of total calories	246±158 (43%)	275±178 (40%)	0.77
Alcohol (ml)	21 standard drinks (1/2 pint of beer, small glass of wine, one measure of spirits)	15±13	14±13	0.98
Monounsaturated fatty acids (g)	>12% of total energy	38±17 (15%)	51±27 (17%)	0.13
Polyunsaturated fatty acids (g)	>6% of total energy	16±7 (6%)	22±13 (7%)	0.20
Saturated fatty acids (g)	<10% of total energy	34±13 (14%)	49±25 (16%)	0.04*
Cholesterol (mg)	300 mg	381±173	469±236	0.13
Total sugar (g)	<10% of total energy	115±67 (20%)	125±76 (18%)	0.81

Starch (g)		128±96	147±104	0.62
Fibre (g)	>25g	19±15	20±15	0.88
Vitamin A (µg)	800 µg	715±577	1505±975	0.005 *
Thiamine (mg)	1.1 mg	1.8±1.2	1.9±1.2	0.87
Riboflavin (mg)	1.4 mg	2.3±1.5	2.6±1.7	0.45
Niacin (mg)	16 mg	27±15	29±19	1.00
Vitamin B6 (mg)	1.4 mg	3.1±3.0	2.9±1.9	0.81
Vitamin B12 (µg)	2.5 µg	7.8±3.6	11±5.8	0.04*
Folate (µg)	200 µg	339±284	328±236	0.58
Vitamin C (mg)	80 mg	104±60	79±41	0.16
Vitamin D (µg)	5 µg	3.6±2.3	5.1±3.5	0.09
Vitamin E (mg)	12 mg	14±7	16.6±9.7	0.41
Phosphorous (mg)	700 mg	1612±763	1787±942	0.64
Calcium (mg)	1000 mg	914±387	1062±548	0.34
Iron (mg)	7 mg	15±11	16±11	0.81
Selenium (µg)	55 µg	67±30	79±43	0.49
Zinc (mg)	10 mg	12±6	13±7	0.58
Sodium (mg)	1600 mg	2983±1559	3385±196 4	0.59
Potassium (mg)	2000 mg	3798±1603	4015±195 8	0.85
Magnesium (mg)	375 mg	359±200	343±189	0.67

Copper (mg)	1 mg	1.2±0.6	1.5±0.8	0.31
Chloride (mg)	800 mg	4407±2407	4885±288 6	0.64
Manganese (mg)	2 mg	3.1±1.5	3.2±1.8	0.85
Iodine (µg)	15 µg	169±76	201±100	0.28

Table 3: Change in food group intake between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Food Group	Pre-course	Post-course	P-value
Red meats	0.62±0.35	0.85±0.56	0.07
Processed meats	0.58±0.77	0.33±0.27	0.08
Poultry	0.31±0.26	0.25±0.22	0.73
Organ meats	0.04±0.07	0.11±0.09	0.01*
Fish	0.55±0.43	0.68±0.49	0.04*
Fried foods	0.21±0.14	0.30±0.16	0.03*
Refined carbohydrates	0.84±0.56	1.28±1.17	0.26
Whole grains	0.66±0.41	0.95±0.75	0.29
Cereal	0.69±1.45	0.47±0.55	0.76
Potatoes	0.34±0.26	0.49±0.34	0.06
Pasta meals	0.42±0.31	0.34±0.25	0.66
High-fat dairy products	2.03±1.15	3.59±3.07	0.09

Low-fat dairy products	0.21±0.27	0.36±0.28	0.02*
Egg dishes	0.61±0.49	0.43±0.29	0.34
Fruit	2.02±1.2	1.38±0.84	0.04*
Green leafy vegetables	0.77±0.42	1.25±1.03	0.11
Cruciferous vegetables	0.68±0.61	0.44±0.32	0.34
Starchy vegetables	0.42±0.56	0.37±0.24	0.16
Other vegetables	3.98±2.17	3.72±2.03	0.66
Legumes	0.30±0.24	0.22±0.25	0.10
Sweets	1.77±1.29	2.45±2.00	0.31
Snacks	0.48±1.00	0.37±0.66	0.40
Soups	1.15±1.24	1.11±1.17	0.48
Sauces	0.19±0.17	0.27±0.28	0.31
Condiments	2.68±1.65	3.17±2.12	0.58
Non-alcoholic beverages	2.39±1.71	1.99±1.42	0.45
Alcoholic beverages	1.43±1.21	1.42±1.29	0.91
Fruit Juice	0.42±0.71	0.39±0.53	0.50
Sweetened beverages	0.81±1.03	0.78±0.74	0.56

Participants intake of milk and dairy products are summarised in Table 4. In relation to participants overall intake of milk, this did not change during the course (mean

increase from 177mls±120 to 192mls±134, $p=0.60$). However, a switch to unpasteurised milk was evident for most participants. Only one of 24 (4%) participants reported consuming unpasteurised milk prior to commencing the residential course while 23 participants (96%) reported its consumption during the course (mean increase from 23±116mls to 239±51mls, $p<0.0001$). Pre-course, 3 participants consumed skimmed milk, 7 semi-skimmed, 11 whole and 3 non-specific and post-course only one participant consumed semi-skimmed while the remaining participants consumed whole milk, consistent with unpasteurized milk intake. Total intake of dairy products (cream, yoghurt, dairy desserts, cheese; salad cream or mayonnaise, butter, cottage cheese) did increase slightly though not to a statistically significant level (mean increase from 2.24±1.23 daily servings to 3.35±3.16, $p=0.07$). Two participants (8%) reported intake of unpasteurised dairy products prior to the course whereas 21 (87.5%) consumed these products during the course (mean increase from 0.01±0.04 servings per day to 1.2±1.4, $p<0.0001$).

Table 4: Change in participants consumption of milk and dairy products between pre-course and post-course time points, obtained from analysis of food frequency questionnaires. (P-values reaching statistical significance are in bold and accompanied by an asterix)

Dairy intake	Pre-course	Post-course	P-value
Total Milk (mL)	177mls±120	192mls±134	0.6
High-fat dairy products (servings/day)	2.03±1.15	3.59±3.07	0.09
Low-fat dairy products (servings/day)	0.21±0.27	0.36±0.28	0.02*
Total dairy products	2.24±1.23	3.35±3.16	0.07

Unpasteurised milk (mL)	23±116	239±51	<0.0001*
Unpasteurised dairy products (servings/day)	0.01±0.04	1.2±1.4	<0.0001*

6.3.3.3 Change in Microbiome composition

We quantified the microbial diversity within each subject (α -diversity) before and after the course, and the difference between each subject's pre-course and post-course gut microbiota (β -diversity). No significant differences were found in either α -diversity (simpson; $p = 0.41$, shannon; $p = 0.26$) or β -diversity ($p = 0.998$) (Figures 6.3-1A and 6.3-1B). No differences were found between males and females.

Analysis of the differential relative abundance of bacterial taxonomic groups revealed a total of 578 amplicon sequence variants (ASVs) within our samples (Figure 1D). Undirected pairwise analysis of all ASVs, (Wilcoxon signed rank test, allowing for Storey's q -value post-hoc correction) revealed only one ASV which changed significantly between pre-course and post-course time points. This ASV corresponded to the genus, *Lactobacillus* which increased significantly ($p=0.0003728$; $q = 0.0498$) (Figure 6.3-1C). Identification of ASVs at a species level was not possible. We subsequently performed a directed search in relation to other lactic acid bacteria (LAB), a dominant population in bovine milk prior to pasteurization based on what was previously reported in the literature on the subject [21]. On the genus level, the relative abundance of *Leuconostoc* ($p=0.09$) and *Enterococcus* ($p=0.14$) did not change but that of *Lactococcus* increased significantly ($p=0.01$; $q = 0.0108$). Other major components of unpasteurized milk include *Pseudomonas* and *Acinetobacter*, We did not detect these genera in any of our samples.

We directly tested for associations between changes in dietary components and changes in microbiome composition within each subject over time. We observed a positive correlation between *Lactobacillus* abundance and combined intake of unpasteurized milk and dairy products (Spearman correlation, $r=0.618$, $p=0.0000027$) Upon closer inspection, *Lactobacillus* abundance appeared to fall into two groups based on the centred log-ratio (clr) transformation of relative abundance scores of >2.5 or <2.5 , prompting us to dichotomize the data for Pearson's Chi-squared test. We defined these groups as low versus high *Lactobacillus* abundance and found a positive association between these two groups and the change in intake of unpasteurized milk and dairy products (combined score), binned into four groups based on the amount of portions consumed; 0-2, 3-4, 5-6 and 7-8. (Pearson's Chi-squared, $X\text{-squared} = 13.265$, $df = 3$, $p\text{-value} = 0.004096$) (Figure 6.3-2). This association also held when looking at the relationship between *Lactobacillus* abundance and unpasteurized milk or unpasteurized dairy products individually. We analysed this *Lactobacillus* grouping against our other metadata (including age, sex, BMI, sleep, exercise and gastrointestinal parameters) but found no other factors associated with the high versus low split.

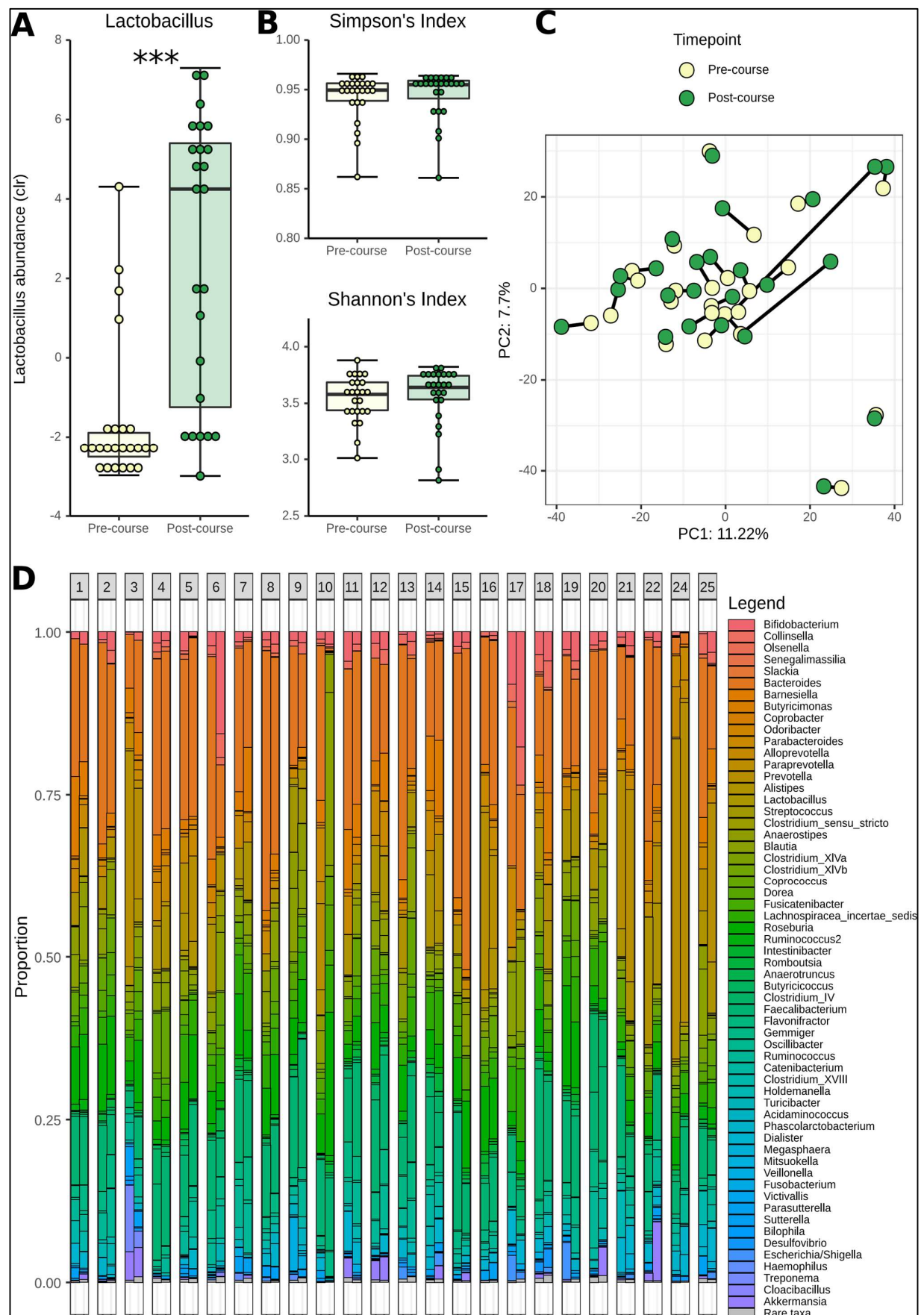


Figure 6.3-1: A; Relative abundance of *Lactobacillus* at pre-course and post-course

time points. **B**; Alpha diversity of gut microbiome at pre-course and post-course time points. **C** Beta diversity of gut microbiome at pre-course and post-course time points. **D**; Relative abundance of genus-level taxa for each participant. Each column represents one participant with pre-course taxa on the left and post-course taxa on the right. (Box plots: Body represents median and interquartile range, whiskers represent the extreme values)

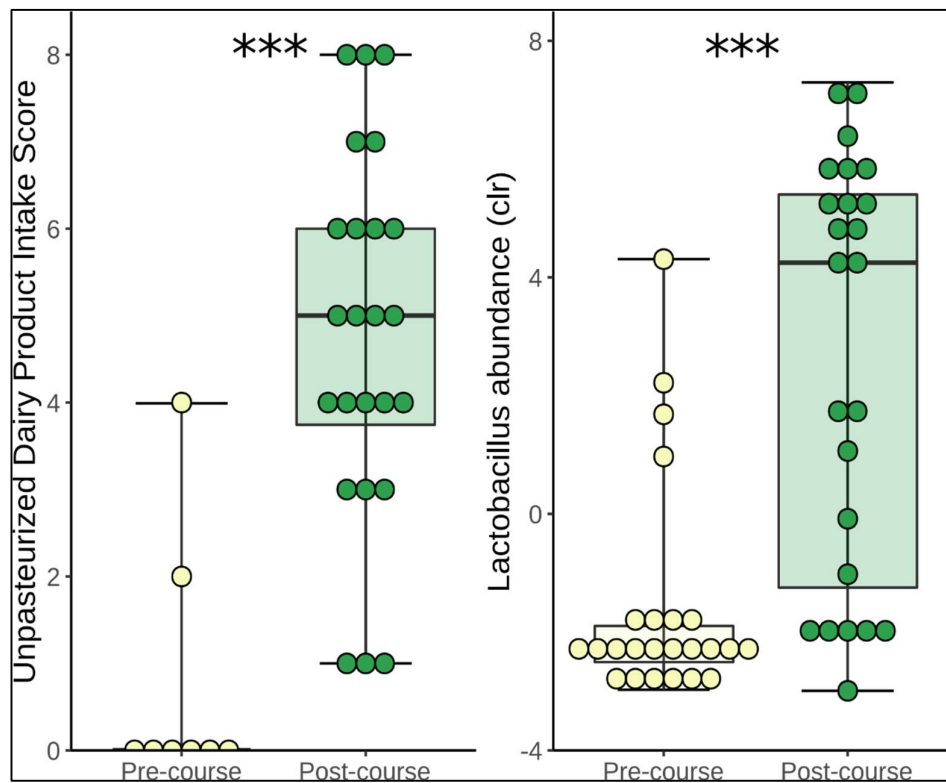


Figure 2: Box plots showing the change in combined unpasteurised dairy score and *Lactobacillus* abundance between pre- and post-course time points. (Body represents median and interquartile range, whiskers represent the extreme values; As some scores overlap, each participant is not visible as an individual point on the graph)

6.3.3.4 Functional Prediction and Application of Gut-Brain Modules

Functional analysis of our microbiome data was performed using Piphillin (Iwai et al., 2016) and further extended by subjecting our metagenomic data to a module-based analytical framework which targets microbial pathways involved in microbiota-gut-brain communication, thus generating a predication of the neuroactive potential of a microbiome sample (Valles-Colomer et al., 2019). Within our sample, we observed 43 of the 56 gut-brain modules (GBMs) described previously by the authors. In addition, we observed an increase in the functional richness of the microbiome profile, as determined by the number of gut-brain modules (GBMs) present (Wilcoxon signed rank test; mean increase of 1.79, $p=0.00087$), following the 12-week course (Figure 6.3-3A). On analysis of the individual GBMs, one consistently increased significantly; GBM026: Nitric oxide synthesis II (nitrite reductase) ($p = 0.001$; $q = 0.061$) (Figure 6.3-3B). Notably, GBM004: Kynurenine synthesis was never found in participants pre-course, but was detected in 6 out of 24 participants post-course at very high levels. This observation did not pass the post-hoc correction ($p = 0.036$, $q = 0.361$) (Figure 6.3-3C). Functional alpha diversity, measured here by calculating the alpha diversity of the floored KEGG Orthologue tables generated by Piphillin, did not differ between pre- and post-course time points (chao1; $p = 0.14$, simpson; $p = 0.19$, shannon; $p = 0.85$).

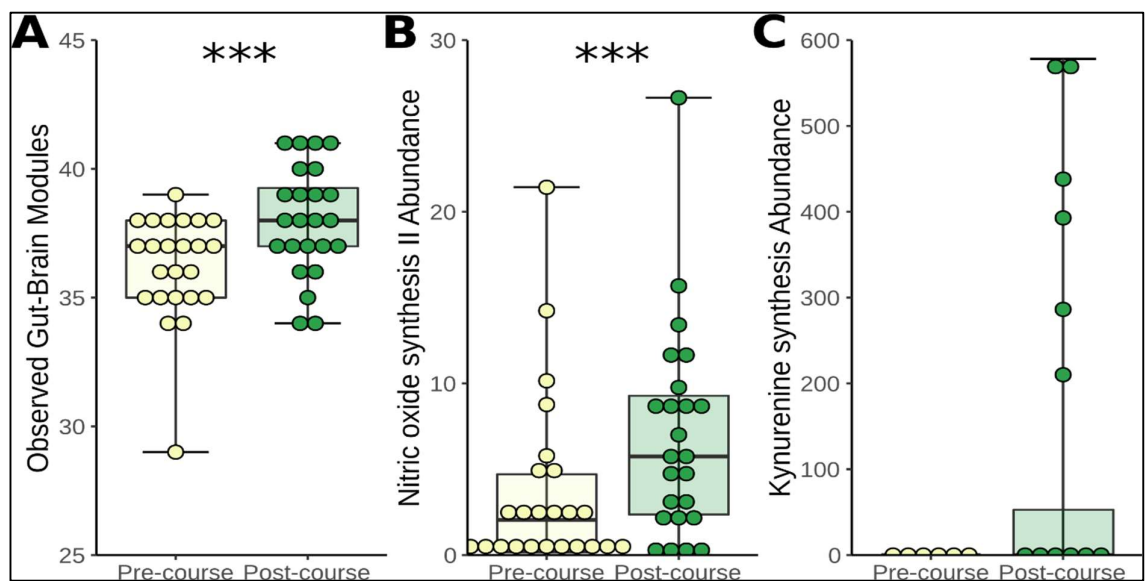


Figure 6.3-3: A: Functional richness of microbiome, as measured by observed number of gut-brain modules (GBM) at pre-course and post-course time points. **B:** Increase in

abundance of GBM 026: Nitric oxide synthesis II (nitrite reductase) between pre- and post-course time points. C: Increase in GBM 004: Kynurenine synthesis between pre- and post-course time points. (Box plots: Body represents median and interquartile range, whiskers represent the extreme values; As some scores overlap, each participant is not visible as an individual point on the graph)

6.3.3.5 Change in microbiome metabolites

Analysis of faecal short-chain fatty acids (SCFAs) revealed a significant increase in Valerate (p=0.04) over the 12 weeks. Propionate also increased, although not to a statistically significant level (p=0.08) while no change was observed in butyrate, iso-butyrate, iso-valerate or acetate. (Table 5)

Table 5: Short-chain-fatty-acid (SCFA) concentrations;

pre- and post-course results

SCFA ($\mu\text{mol/g}$)	Pre- course Mean (SD)	Post- course Mean (SD)	P- value
Acetate	27.0 (8.6)	29.3 (10.3)	0.268
Propionate	14.0 (7.0)	16.3 (7.6)	0.091
Iso-butyrate	2.4 (1.1)	2.6 (0.9)	0.485
Butyrate	17.6 (9.6)	19.0 (10.8)	0.156

Iso-valerate	3.2 (1.9)	3.4 (1.7)	0.498
Valerate	2.3 (0.8)	2.5 (0.8)	0.049
			*
Total BCFA	5.6 (2.9)	6.0 (2.5)	0.44
Total SCFA	66.6 (24.7)	73.1 (27.7)	0.113

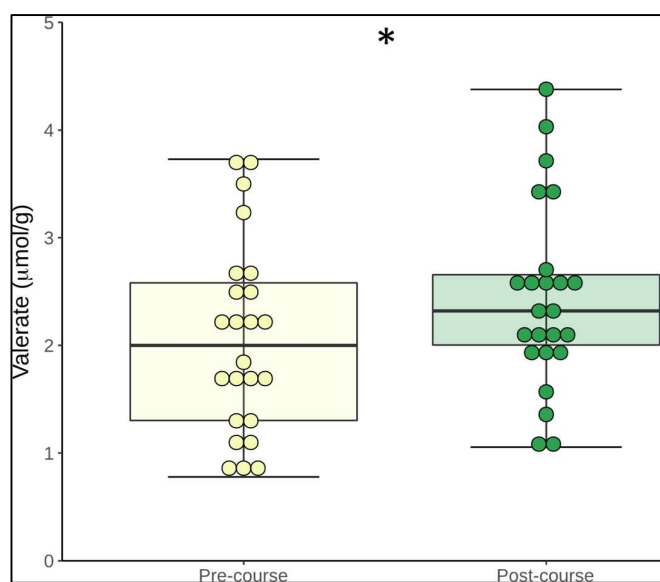


Figure 6.3-4: Concentration of Valerate at pre- and post-course time points. (Body represents median and interquartile range; whiskers represent the extreme values)

6.3.3.6 Change in psychological measures

There was no change in total scores on the Cohen's Perceived Stress Scale (PSS), Hospital Anxiety and Depression Scale (HADS)-total score, HADS-anxiety subscale or HADS-depression subscale between pre- and post-course time points. However, because our study involved a healthy population, baseline anxiety and stress scores were low and mood scores were within the normal range (Table 6). Further analysis was considered taking into account baseline scores. The sample was dichotomized based on the median score of the above scales. Participants with higher baseline scores on the PSS showed a mean reduction of 4.42 points, whereas the rest of the participants reported a mean increase of 1 point (Wilcoxon test, $p=0.026$) between pre-course and post course time points. Participants with higher anxiety scores than the median on the HADS-A also showed a statistically significant reduction compared to the rest of the participants (mean reduction of 2 vs a mean increase of 2, Wilcoxon test $p=0.0043$). We did not find any relationship between microbes and psychological scales. No differences were found between males and females.

Table 6: Results of psychological scales at pre- and post-course time points

Scale/Mean (SD)	Pre-course	Post-Course	p-value
PSS	14.96 (6.23)	13.13 (5.12)	0.149
HADS-A	5.61 (3.72)	4.83 (3.21)	0.274
HADS-D	3.04 (2.82)	3.83 (3.42)	0.198
HADS-T	8.65 (5.37)	8.65 (5.93)	1

HADS: Hamilton Anxiety and Depression Scale, HADS-D: HADS-Depression subscale, HADS-A:

HADS-Anxiety subscale, HADS-T: HADS-Total. PSS: Perceived Stress Scale

6.3.4 Discussion

In this observational study, we investigated the effect of a dietary change on the gut microbiome of participants who undertook a 12-week residential cookery course on an organic farm, where the majority of food consumed and used for cooking, was locally-sourced, seasonal and produced using organic methods. Of particular interest was the use of unpasteurized milk and dairy products obtained from a small herd of Jersey cows on the farm. Most participants had not been using any unpasteurized dairy prior to the course and all used these products to some degree throughout their stay. We found that the main change in terms of microbiome composition was a dramatic increase in participants *Lactobacilli* between pre-course and post-course faecal samples. This increase was strongly associated with participants intake of unpasteurized milk and dairy products. In addition, a positive change was noted in relation to microbiome metabolites with an increase in valerate and, to a lesser extent not quite reaching statistical significance, propionate.

While administration of probiotics in the form of conventional pharmaceutical agents such as tablets or capsules is a common method, the majority of probiotics commercially available are in the form of food-based delivery systems which use probiotic bacteria in their production or add these bacteria during the manufacturing process, e.g., cheese, yoghurt or fermented drinks (Govender et al., 2014). There are several problems associated with pharmaceutical and commercially-produced probiotic formulations. Firstly, the probiotic potential of bacteria is species and strain-specific but efficacy is often generalized across products in the current unregulated commercial probiotic market (de Simone, 2019). Secondly, there are many aspects of the manufacturing process of such products which can alter the delivery of viable functional probiotic bacteria (Sanders et al., 2014). Because probiotic products are generally categorized as food supplements, they are subject to less stringent regulatory criteria and quality control processes with regard to microorganism specification, their numbers and functional properties (Kolacek et al., 2017). Thirdly, there is a cost consideration when it comes to commercial probiotic products, which may place daily probiotic supplements out of the reach of many.

An alternative to consuming commercially-produced probiotic supplements for the maintenance of a healthy gut microbiome is to alter one's diet. It is increasingly accepted that the 'Western-diet', characterized by highly-processed, low-fibre, high-sugar, high-fat foodstuffs has negative implications for health (Cordain et al., 2005) which may be mediated by an unfavourable impact on the gut microbiome (Zinocker and Lindseth, 2018). In contrast, adherence to a Mediterranean-style diet (characterized by high-level consumption of olive oil, fruit, nuts, vegetables, and cereals with moderate intake of fish and poultry) has been strongly associated with better physical (Estruch et al., 2018) and mental (Lassale et al., 2018) health outcomes, which again may be related to a beneficial impact on the gut microbiome and metabolome (De Filippis et al., 2016). Gut microbiome composition can be rapidly and significantly altered by introducing dietary change (David et al., 2014) with the impact of food choices on the microbiome being highly individualized (Johnson et al., 2019). In this study, the key change in relation to dietary intake during the 12-week residential course was an increase in dairy products, which in this context were unpasteurized. This was a major change for our subjects, the vast majority of whom did not consume unpasteurized milk or dairy products prior to the course.

Cow's milk is produced on a massive scale worldwide and has long played an important role in human nutrition (Haug et al., 2007). Cow's milk harbours a rich microbiota and typically contains a significant population of lactic acid bacteria (LAB) that includes *Lactococcus* (8.2×10^1 – 1.4×10^4 CFU/ml), *Streptococcus* (1.41×10^1 – 1.5×10^4 CFU/ml), *Lactobacillus* (1.0×10^2 – 3.2×10^4 CFU/ml), *Leuconostoc* (9.8×10^1 – 2.5×10^3 CFU /mL) and *Enterococcus spp.* (2.57×10^1 – 1.58×10^3 CFU/mL) (Quigley et al., 2013). Other organisms present in substantial proportions are *Pseudomonas* and *Acinetobacter*, so-called psychrotrophs which can flourish during cold storage conditions and typically cause milk spoilage (Raats et al., 2011). Pasteurization of milk gained widespread popularity in the early 1900's when cow's milk was linked to the spread of disease epidemics such as tuberculosis, diphtheria, typhoid fever, scarlet fever, anthrax and cholera (Rankin et al., 2017). A recent Irish study, using molecular, culture-independent techniques, compared the microbial content of raw and

pasteurized cow's milk (Quigley et al., 2013). Authors reported that, although the bacterial diversity of the raw and pasteurized milk were similar, raw milk contained mostly viable cells whereas the cell population in pasteurized milk were predominantly non-viable. Thus, while pasteurized milk appeared to have a somewhat similar microbiome composition to that of the raw milk, any potential probiotic LAB would have been in a nonviable state. In this study, *Pseudomonas* and *Acinetobacter*, two major genera found in unpasteurized milk, were not detected by 16S rRNA analysis of the microbiomes of the participants, either pre or post treatment. This may be due to a selective filtering effect of the human immune system or physiological barriers such as gastric acid, which is known to act as such a filter (Freedberg et al., 2015; Imhann et al., 2017).

The consumption of raw milk is growing in popularity, although there is some debate in relation to its purported benefits and concern about the potential dangers of contracting milk-borne illnesses if the raw milk is contaminated with human pathogens (Lucey, 2015). There is a strong suggestion from epidemiological literature that the consumption of unpasteurized cow's milk or yoghurt by children living on farms or rural areas has a protective effect against the development of asthma, allergies and atopy, a finding which seems to be independent of other farm-related exposures (Braun-Fahrlander and von Mutius, 2011). In addition, raw milk is anecdotally reported to be beneficial for people with lactose intolerance (Beals, 2008). This is thought to be due to the fact that raw milk contains high counts of LAB that produce lactase enzymes, which would otherwise be destroyed during pasteurization. However, there is little research evidence to support these anecdotal claims and, in fact, one recent pilot randomized controlled trial (RCT) involving 16 adults with lactose malabsorption, failed to find any benefit of raw milk over pasteurized milk for gastrointestinal symptoms (Mummah et al., 2014). Despite this, in a survey of raw-milk consumers (Mullin and Belkoff, 2014), over one-third of responders claimed to experience gastrointestinal discomfort from drinking pasteurized milk but no discomfort after drinking raw milk, although the vast majority of these people did not have a diagnosis of lactose intolerance. Another proposed benefit of raw milk is that it contains higher quantities of vitamins. A meta-

analysis (Macdonald et al., 2011) reported that pasteurization reduced the concentrations of Vitamin E, Vitamin B12, Vitamin B2, Vitamin C and folate. Of these vitamins, B2 is of most importance as bovine milk contributes significantly to the recommended daily intake whereas in the case of all the others, milk is not typically an important source. In relation to the human gut microbiome, we are unaware of any studies specifically examining the effect of raw milk consumption. However, a few studies have investigated the impact of pasteurized milk on the human microbiome. One cross-sectional study reported a differential oral microbiome based on high versus low (pasteurized) milk intake (Johansson et al., 2018). Another investigated the impact of whole milk supplementation on the gut microbiota and cardiometabolic biomarkers between lactose malabsorbers (LM) and absorbers (LA) (Li et al., 2018). Authors found that whole milk supplementation significantly altered the intestinal microbiota composition in LM resulting in an increase in the phylum Actinobacteria along with increases in several genera; *Bifidobacterium*, *Anaerostipes* and *Blautia*. These changes occurred only in LM and not LA, suggesting that it was the increased lactose substrate reaching the colon which preferentially enhanced the growth of some micro-organisms. In addition to pasteurization, milk can be altered by skimming which is currently a widespread procedure. Prior to the course 10/24 of our participants reported consuming skimmed or semi-skimmed milk while post-course 23/24 participants consumed whole milk, reflecting the unpasteurized milk intake. Skimmed milk contains less fat than whole milk and thus also less fat-soluble vitamins such as A and E. However, regular unfortified milk is not a major contributor to a person's recommended daily allowance of these vitamins (Herrero et al., 2002) and despite the variable amounts in different milk types there does not appear to be significant difference in their bioavailability (Herrero-Barbudo et al., 2006). Other micronutrients such as calcium, sodium and choline do not differ between skimmed and whole milk (Manzi et al., 2013). Therefore, we considered the skimmed versus whole milk type to be of limited consequence.

An obvious limitation of this study is the inherent potential for confounding given that, in addition to a change in diet, study participants experienced a change in environment. Disentangling the impact of diet and geographical environment on the

gut microbiome, however, is a very difficult task. Several large scale studies have attempted to explore the differences in microbiome composition between industrialised Western urban dwellers and those living in traditional rural communities in South America and Africa, such as the Hadza hunter-gatherers of Tanzania (Schnorr et al., 2014), rural Papua New Guineans (Martínez et al., 2015) children from the rural African village of Burkina Faso (De Filippo et al., 2010) and communities from Malawi and Amazonian Amerindians (Yatsunenko et al., 2012). Although a rural setting will likely contribute to gut microbiome differences, these farming environments are intrinsically linked to variation in diet and it is difficult to separate the impact of the farm environment itself and the farm-related dietary patterns. If a move to a rural farming environment were to account for the changes in microbiome seen in our study one could postulate that the changes would be consistent with the microbiome composition in rural dwellers from the above studies. This was not the case. While rural dwellers from PNG did have higher abundance of *Lactobacillus* than their urban counterparts (Martínez et al., 2015), those from the other rural farming communities did not (De Filippo et al., 2010; Schnorr et al., 2014). Obviously, the rural locations in the above studies were at the extreme end in relation to geographical location and traditional lifestyle and poorly comparable to the developed farm environment in which our participants were based. In a study more closely resembling our location, authors compared the microbiome of infants from farming and non-farming families in Wisconsin, United States, and again no differences in *Lactobacillus* or other LAB abundance were seen (Thorsen et al., 2019). Furthermore, the changes in bacterial taxa in the microbiome of our subjects were consistent with those species found in unpasteurized milk, supporting our conclusion that this specific dietary change was driving the microbiome differences between pre- and post-course time points.

In this study we found that, during the 12-week course, the levels of the faecal SCFA valerate increased with a trend towards increase in proprionate. Straight-chain SCFAs (acetate, butyrate, propionate and valerate) are produced by the gut microbiota during the fermentation of partially and nondigestible polysaccharides whereas branched-chain SCFAs (isobutyrate and isovalerate) result from the metabolism of

proteins (He et al., 2018). SCFAs are thought to play a major role in the maintenance of gut and immune homeostasis (Tan et al., 2014) as well as in the gut-brain axis response to stress (van de Wouw et al., 2018). SCFA production can be stimulated by increasing dietary fibre intake (Francois et al., 2012) or protein consumption (Russell et al., 2011). However, in our study, participants intake of fibre or protein did not change and thus, it is proposed that increased valerate and propionate levels may have been secondary to increased abundance of *Lactobacilli*, which, along with other LAB, are known producers of SCFA (LeBlanc et al., 2017). Propionate has anti-inflammatory properties and has been shown to be of potential benefit across a range of disorders, including hypertension and cardiovascular disease (Bartolomeus et al., 2019), obesity (Chambers et al., 2015) and hypercholesterolemia (Demigne et al., 1995). Valerate is a less well-known SCFA with limited research to date into its therapeutic potential. However, a recent study revealed that it also appears to have an immunomodulatory effect (Luu et al., 2019). Interestingly, supplementation with *Lactobacillus acidophilus* increased the concentration of valerate in the caecum of chickens infected with *Clostridium perfringens* while reducing the infection-associated gut dysbiosis (Li et al., 2017). Valerate may also hold some translatable therapeutic value in the context of *Clostridium difficile* infection (CDI). Valerate was shown to be significantly reduced in the faecal samples of patients with recurrent CDI and recovered following successful treatment with FMT (McDonald et al., 2018).

Changes in the functionality of the microbiome were assessed in the context of a recent study which facilitates analysis of the neuroactive potential of a microbiome sample (Valles-Colomer et al., 2019). Authors achieved this using a gut-brain-module (GBM) framework which targets microbial pathways known to be involved in microbiota-gut-brain communication and have made this GBM catalogue available for use by other researchers (<https://raeslab.org/software/gbms.html>). When applying our predictive metagenomic data to this GBM catalogue we found an increase in the functional richness of the microbiome profile, as determined by the number of GBMs present, following the 12-week course (Figure 4). Such a consistent general increase in GBMs without a significant increase in microbial alpha diversity goes somewhat against the intuition that a more diverse microbial ecosystem will

necessarily display a higher functional diversity. More strikingly, the functional alpha diversity did not change during the course. GBMs represent a specific subset of microbiome function and are calculated using the values of specific KEGG Orthologues. A shift in microbial functions that specifically potentially impact the host brain without a corresponding general shift in microbial function detectable on the alpha diversity level shines light on the possibility that many more such specific shifts can occur undetected using current bioinformatics tools. Because of this, we call for a move away from general diversity and towards informed interrogation of specific functional changes in the microbiome as a readout.

One GBM changed significantly after post-hoc correction; 'GBM026; Nitric oxide synthesis II (nitrite reductase)'. Several studies have demonstrated the ability of various *Lactobacillus* species to synthesize nitric oxide by nitrate reductase activity (Xu and Verstraete, 2001; Liu et al., 2014). Nitric oxide is a complex and widespread signaling molecule which participates in virtually every organ system of the body. It is thought to play a role in the stress response and mood regulation (McLeod et al., 2001) and may represent one mechanism by which *Lactobacilli* exert psychobiotic effects. The authors believe another GBM warrants discussing here, although its increase did not satisfy significance after post-hoc correction; 'GBM004, Kynurenine synthesis'. This module was never detected in participants pre-course but was present in very high levels in 6 out of 24 participants post-course. This can be explained by the fact that the Kynurenine synthesis module requires two enzymatic steps. One of these was found in *Lactobacillus*, but the other one was not specific to a single microbe in this data set, but rather spread over several microbes and was only found in the 6 participants positive for MBG004. This finding conforms well with literature regarding emergent biosynthetic capacity of the microbiome (Chiu et al., 2014; Perisin and Sund, 2018).

Although we found no direct correlation between *Lactobacillus* abundance and psychological measures, it is notable that stress and anxiety levels reduced significantly in those with higher baseline scores on the PSS and HADS-A. This is consistent with probiotic interventional trials in healthy populations, whereby an impact is often only seen in those with higher anxiety or depression scores at baseline

(Ng et al., 2018; Liu et al., 2019). Of course, there are many possible confounding factors when it comes to interpreting this reduction. Participants in this course had varying reasons for completing the course; for some, the purpose was to enhance or change their career options and thus, possibly associated with stress; for others it was simply for leisure and viewed more as a holiday incorporating cookery classes. The change in environment and daily activity, the purpose of participation in the course and interaction with new people may all have contributed to psychological status. However, given the increasing evidence that the gut microbiome is an important node in gut-brain communication and that certain psychobiotics have anxiolytic effects, it is plausible to consider the possibility that the improvement in stress and anxiety may have been partially related to the increase in *Lactobacillus*. *Lactobacillus rhamnosus* (JB-1) has been shown to reduce anxiety behaviours in mice as well as altering central levels of gamma-aminobutyric acid (Bravo et al., 2011), a key neurotransmitter in anxiety regulation. Several species of *Lactobacillus* have demonstrated the ability to reduce anxiety and stress levels in healthy subjects (Messaoudi et al., 2011; Nishihira J. et al., 2014; Takada et al., 2016) as well as in patients with chronic fatigue syndrome (Rao et al., 2009) or laryngeal cancer (Yang et al., 2016).

There are several limitations to our study. Firstly, this was an observational study. While of course an RCT would be preferable, there are many challenges inherent in designing RCTs involving dietary interventions. It can be difficult to define appropriate control groups and effective blinding of participants and investigators is often extremely difficult (Weaver and Miller, 2017). In particular, it can be challenging to accomplish a high level of adherence with whole food, or dietary pattern, interventions. A major strength of our study in this regard was that our participants were based on-site for the entire duration of the study making it possible to ensure a consistency across individual diets which would be difficult to achieve outside a residential setting. The potential confounding effect of the farm environment as an independent modulator of microbiome composition is addressed earlier in the discussion. Secondly, our sample size was quite small. However, previously published studies investigating the diet-microbiome relationship have

involved participant numbers of ten or less (David et al., 2014; Ruggles et al., 2018) and have generally been of much shorter duration (Johnson et al., 2019). Another factor which may limit the generalizability of our study was that participants undertaking this course were interested in food and cooking. Thus, they were likely to have good nutritional knowledge and possibly healthier than average diets at baseline. A specific limitation in this regard was an absence of any information on the use of non-nutritive sweeteners (NNS). These are being increasingly used due to the concern about the negative health impact of high-sugar diets and have been shown to significantly, and generally negatively, impact the gut microbiome (Suez et al., 2015). Finally, given the limitations of 16S rRNA gene sequencing we were unable to characterize organisms beyond the genus level. More accurate taxonomic classification would have been useful had shotgun metagenomic sequencing been performed. Despite these limitations, this is, to our knowledge, the first study to report on the potential impact of unpasteurized milk and dairy products on the human gut microbiome. Given the growing popularity of consumption of raw milk and other probiotic-rich fermented foods, it is important that the effect of such products on the gut microbiome are investigated.

While there are understandable concerns in relation to potential contamination and safety when it comes to unpasteurized milk, it is a rich source of probiotic bacteria. Abundances of *Lactobacilli* increased significantly following a 12-week dietary change which involved consumption of unpasteurized milk and dairy products. *Lactococcus* abundance, also increased, although to a lesser extent. These changes in microbiome composition were reflected by an increase in levels of the SCFA, valerate with an observed trend towards increase of propionate, along with an increase in the predicted functional richness of the microbiome. Given the growing appreciation of the importance of a healthy gut microbiome and the limitations of commercial probiotic products, there is a need for further research into the effect of different dietary changes on the microbiome. In particular, further studies investigating the probiotic potential of natural probiotic-containing foodstuffs such as unpasteurized milk are warranted.

6.3.5 Methods

6.3.5.1 Study Site and Subjects

Ballymaloe Cookery School, Organic Farm and Gardens is located in East Cork, Ireland and runs bi-annual 12-week residential courses where students live on-site, learn about organic farming methods and undertake intensive cookery classes. The majority of food consumed by participants during their stay originates from the organic farm itself or consists of high-quality, locally-sourced produce. The farm has a small herd of Jersey cows whose milk is used in its raw unpasteurized state for direct consumption, cooking and the production of other dairy products including cream, butter, cheese and yoghurt. There is an emphasis on eating, and cooking with, local seasonal fruit and vegetables, the vast majority of which is organic. Meat and fish are also locally sourced and, for the most part, organic.

Approval of the study protocol was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Protocol number DOP001) and conducted following the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. Written informed consent was obtained from all subjects before study procedures were conducted. Course participants were emailed in advance informing them of the study and a short talk on the gut microbiome was given at an introductory session prior to commencement of the course. In order to be eligible for the study, participants had to be between the age of 18-65 years and be generally healthy, with no chronic or current, physical or mental illness. Exclusion criteria included the use of medications which were likely to interfere with the objectives of the study (including any psychotropic medications) as well as intake of antibiotics, probiotics or prebiotics within the month prior to commencement of the study.

6.3.5.2 Subject Metadata

Demographic data was collected for each individual including information on age, sex and race. Weight and height were measured and used to calculate body mass index (BMI). Information in relation to medical and psychiatric history, along with medication use, was also obtained at interview. At the beginning and end of the 12-week course participants completed self-report questionnaires on a variety of

parameters including mood, anxiety, sleep, exercise (PSS, HADS, PSQI, International Physical Activity Questionnaire (IPAQ)).

6.3.5.3 Diet Quantification

To monitor nutrient intake, participants completed the self-administered 152-item SLAN-06 (Survey of Lifestyle, Attitudes and Nutrition in Ireland) food frequency questionnaire (FFQ) (Harrington J, 2008) which is validated to be used in an Irish population. An additional eight food items as well as questions about type and frequency of milk, salt and fried food consumption were added. These items are included in the EPIC Norfolk questionnaire (Riboli and Kaaks, 1997) from which the SLAN-06 FFQ was adapted. An extra section was added to the FFQ by the authors to quantify intake of unpasteurized milk and dairy products before and during the course, as this information would not otherwise have been captured. These extra questions followed the same response format as the other food items.

Participants were asked to estimate the frequency with which they consumed a specified portion size of each of the foods listed over the preceding month. The FFQ has nine possible responses ranging from “never or less than once per month” to “6+ per day”. Participants completed the FFQ before and after the stay at Ballymaloe. The FFQs were analysed for nutrient intake using the FETA software (Mulligan et al., 2014).

The 160 foods items were grouped into 29 food groups (e.g., fruits, vegetables, grains, sweets) using methods similar to those described in previous studies of dietary patterns (Arthur et al., 2013). To estimate the number of servings of any food group, each response was converted to the corresponding frequency factor and summed over all the food items to get the average servings of a specific food group per day. Intake of unpasteurised milk and dairy products was analysed in a similar way.

6.3.5.4 Faecal sample collection and 16S rRNA gene sequencing and processing

Faecal samples were collected at the beginning and end of the 12-week period in disposable plastic containers with a Thermo Scientific™ Oxoid AnaeroGen 2.5L Sachet in situ to generate anaerobic conditions within the container. Participants were

instructed to keep the sample containers in a refrigerator at 4°C. Samples were collected and transferred to a -80°C freezer within 12 hours.

DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen) using the protocol for Gram positive bacteria and including an additional bead beating step at the beginning of the procedure. DNA was quantified using the Qubit High Sensitivity Kit (Life Technologies), standardized and then used as a template for PCR. 16S metagenomic libraries were prepared using primers to amplify the V3-V4 region of the bacterial 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16 s Metagenomic Library Preparation guide. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. The pooled libraries were assessed using an Agilent high sensitivity DNA kit and examined by quantitative PCR (qPCR) using the Kapa Quantification kit for Illumina (Kapa Biosystems, USA) according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced (2 × 300 bp) on the Illumina MiSeq platform.

6.3.5.5 Sequence table generation

Three hundred base pair paired-end reads were prefiltered based on a quality score threshold of > 28 and trimmed, filtered for quality and chimeras using the DADA2 library in R (Callahan et al., 2016). Taxonomy was assigned with DADA2 against the SILVA SSURF database release v132. Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. ASVs were cut off at genus level, those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as non-zero in five percent or fewer of total samples.

6.3.5.6 Short chain fatty acid (SCFA) measurements

The concentration of SCFAs, acetate, propionate, Iso-butyrate, butyrate, Iso-valerate, and valerate were analyzed by gas chromatography flame ionization detection (GC-FID) using a Varian 3800 GC system, fitted with a 5m guard column (Restek) connected to an Agilent DB-FFAP column (30 m L x 0.32mm ID x 0.25 µm df) and a flame ionization detector with a CP-8400 auto-sampler.

6.3.5.7 Statistical analysis

Statistical analysis for changes in dietary measures was performed using SPSS Statistical Packages version 25 (SPSS, Inc., Chicago, IL, USA). Normality of outcome measures was assessed using Shapiro Wilk's test of normality. Differences in nutrient and food group intake pre- and post-course participation were analyzed using Student's t-test or the non-parametric Wilcoxon Rank sum test.

Microbiome data-handling was done in R (version 3.6) with the Rstudio GUI (version 1.2.1555). In all cases, the iNEXT library was used to calculate alpha diversity (Hsieh et al., 2016).

Principal component analysis (PCA) was performed on centered-log ratio transformed (clr) values using the ALDEx2 library (Fernandes et al., 2014). Number of permutations was always set to 1000. Aitchison distance was used as a distance metric for beta-diversity. Piphillin (Iwai et al., 2016) was used for functional inference from 16S rRNA gene sequences of stool samples in the form of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues. Gut-brain modules were calculated using the R version of the Gomixer tool (Darzi et al., 2016). Differential abundance of microbes between groups was assessed using the ALDEx2 library. As part of testing for correlations between microbial abundance and metadata, skadi, an implementation of jackknifing and Grubb's test, was used to assess the reliability of the data and detect outliers (R scripts available online, <https://github.com/thomazbastiaanssen/Tjazi>; (Bastiaanssen T, 2018)). Correlation was assessed using Spearman's rank correlation coefficient. The relationship between categorical variables was assessed using Pearson's Chi-squared test. For datasets in which the condition of normality was violated the non-parametric Kruskal-Wallis test was used and post-hoc analysis was done using the Wilcoxon test. A p-value of < 0.05 was deemed significant. To correct for multiple testing in tests correlating volatility and specific microbiota, KEGG orthologues or pathways, the Q-value post-hoc procedure was performed with a q-value of 0.1 as a cut-off (Storey J, 2019).

Chapter 7 Discussion

7.1 In summary

The microbiome-gut-brain axis field currently spans both clinical and animal research. This is reflected in the approaches used in this thesis, where the chapters were organised into three general categories. It is clear that diet is one of the main ways in which the microbiota can be modified. In chapter 2 of this thesis, which focuses on diet-based animal studies, we found that we were able to blunt the neuroinflammatory effects of ageing in the host by targeting the microbiome, using dietary prebiotic approaches. Similarly, we were able to show that the effects of stress could also be rescued by prebiotics or a dietary supplementation. Notably, not all stressors are created equal and the microbiome does not seem to hold the key in all cases. We were unable to find evidence of rescuing the effects of chronic hypoxia, which was previously shown to alter the microbiome, by prebiotics supplementation. In all cases, altering diet was shown to consistently change both microbiome composition and relative abundance of microbiome features. Importantly, in the last paper in this chapter, we show that is also possible for a poor quality diet such as a high-fat diet or even cafeteria diet to affect the microbiome and its host negatively.

Chapter 3 features four papers and is themed around the effects of disrupting the normal microbiome colonization and homeostasis. It is clear that perturbing the microbiome can affect host brain and behaviour. First, we show that disrupting the microbiome with antibiotics in adolescence has persistent negative effects for host brain and behaviour in adulthood, even though the microbiome itself seems to recover. Then, we show that antibiotics perturbation can alter the permissivity to colonization of new species, potentially allowing the microbiome to develop in ways that would not occur without antibiotics administration, including on a functional level. Continuing this investigation of the effects of perturbing the microbiome, in the next paper we show that the microbiomes from mice delivered by caesarean section are distinct from those delivered vaginally. Like was the case with antibiotic-

based perturbation, the microbiomes converge over time, making them indistinguishable by birth mode, but behavioural effect remain.

Chapter 4 consists of a single paper where we show that deer mice, which naturally tend to compulsive-like behaviour in the form of building, have a different microbiome composition based on their behavioural phenotype.

Then, in chapter 5 we discuss a single paper that not only reconfirms the microbiome-gut-brain axis, but also sheds light on our findings regarding the importance of microbiome perturbation in the previous chapter of this thesis. We investigate the dynamics of microbiome change over time, which is termed volatility. First, we demonstrate how volatility can be measured. Then, we show that volatility is correlated to the severity of stress in both mice and humans. Finally, we show that there are consistent functional changes in the microbiomes of both humans and mice undergoing stress.

In chapter 6 we make the step resolutely from animals to humans. While there are many differences between animal and human research, indeed, one of the many reasons why animal research is so important is the possibility to control for otherwise difficult to control factors like diet, genetics and environment, our findings in this chapter echo what we previously reported in animal work. While there are important differences between the rodent and human research described here, there are interesting patterns and commonalities in the findings worth discussing further. In these three subchapters, we show that we can improve host mood and behaviour by targeting the microbiome, by using a prebiotic in the first paper, a probiotic in the second and a diet rich in unpasteurized dairy products in the third. In order to help the reader link chapters to the sub-aims I introduces in chapter 1.3.3, I have provided a visual aid (figure 7.1-1).

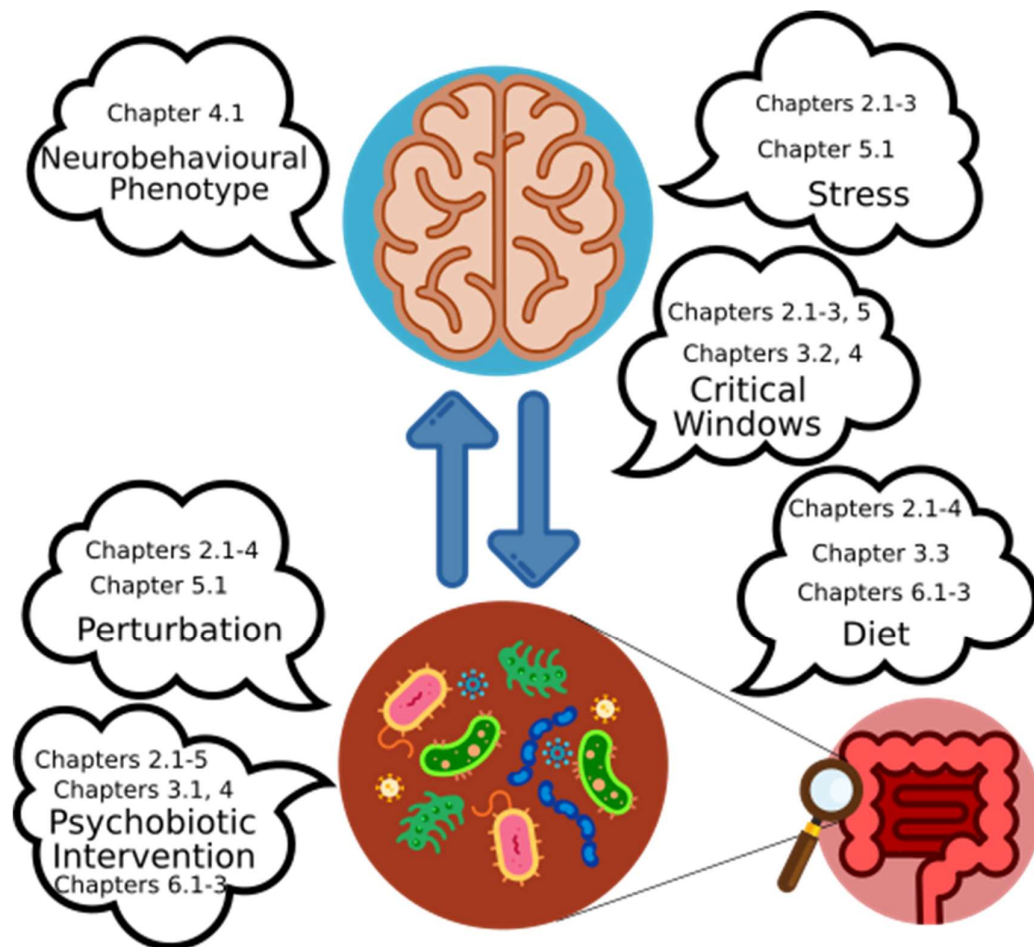


Figure 7.1-1 summarising the aspects of the microbiota-gut-brain axis that have been investigated in the chapters in this thesis. Chapters are depicted in their respective bubbles.

7.2 Identifying patterns in our microbiome-gut-brain axis studies

7.2.1 Enduring effects of perturbations in the microbiome and its lasting effects on the host

The perturbation of the host microbiome is a common pattern among the research described in this thesis. In chapter 2.5 we examined the effects of dietary perturbations in adolescence on microbiome composition in adulthood. Analogously, in chapter 3.2, we used antibiotics to perturb the microbiome in the same stage of life. In both cases we found that, while the microbiome is able to recover on a

compositional level to the extent where it is indistinguishable from controls, lasting effects remain in the host organism. In chapter 3.4, we again find that the differences animals delivered vaginally or by c-section are most clear on the level of the microbiome in early life, but that these differences resolve in adolescence. Again, we find that behavioural effects remain even after the microbiomes have converged. Interestingly, we were also able to show that the conditions of the microbiome can also be such that changes in the microbiome will be lasting. In chapter 3.3, we found that *Blautia* was only able to colonize animals that were given prebiotics in the case that their microbiome was first perturbed by antibiotics. This change was not detected in beta diversity as only a single genus was affected by it, though we were able to show that this increase in *Blautia* would also likely have functional consequences. As past states seem to matter for how a microbiome turns out even if the current environment is constant, one wonders whether the microbiome has a memory and, if so, how we could read it. This also reinforces that time is an important factor when it comes to the microbiome-gut-brain axis. There is evidence of critical windows in the developmental period where the host may be particularly vulnerable to perturbations in the microbiome. Interestingly, the development of the microbiome has recently been shown to follow some consistent and predictable trajectory (Chng et al., 2020; Gibbons, 2020). This taken together with the finding that the infant microbiome strongly selects for specific bacterial taxa from the mother (Ferretti et al., 2018) suggests that the early-life microbiome may provide some metabolic function that is crucial for healthy development of the host. Indeed, as eukaryotes have never existed without prokaryotes, it makes sense from an evolutionary perspective that certain developmental processes in the host organism could rely on the presence of a microbiome with particular metabolic properties.

7.2.2 There is disagreement between studies which specific genera react to stress interventions targeting the microbiota.

Several studies discussed here examine the effects of stress on the microbiome. In all cases, we report changes in microbial genera after stress. However, perhaps surprisingly, the genera that were altered differ between studies. Indeed, chapter 5.1, on volatility, features two cohorts of mice undergoing chronic social defeat

stress, but there is little to no agreement in what genera are affected by this stressor. Similarly, there was little agreement between the specific taxa that were altered in chapter 2.4, where we rescue the effects of chronic stress. In that study we also measured the microbiome in response to stress, albeit in rats this time rather than mice.

There are several reasons for this disagreement, some of which lead to interesting hypotheses and points of discussion. First, we should acknowledge the consistent biases that are inherent to modern metagenomic analysis (McLaren et al., 2019). As sequencing efficiency has been shown to differ between laboratories and even between researchers or individual plates to be sequenced, we cannot assume that the same microbiome perturbation will give the same signature between two experiments. Second, as is likely the case for the volatility study in chapter 5.1, the microbiome compositions at baseline can easily differ between cohorts of animals. It stands to reason that if conditions in the microbiome at baseline are different between two cohorts, one cannot expect the same treatment to have the same effect there.

This makes the finding that there is indeed concordance in the stress response in the microbiome on an inferred functional level in these aforementioned animals from the volatility study in chapter 5.1 particularly striking. Indeed, it has been reported that there is more unexplained variance on the taxonomical level of the microbiome than on the functional level, suggesting that function is more strongly conserved than taxonomy in the microbiome (Eng and Borenstein, 2018; Mehta et al., 2018). This stands in contrast with the strict strain-level selection during early life development we discussed in the previous point. In both the cases of stress and development do we see an agreement on a functional level, but we only see agreement on the functional level in the case of early life development (Ferretti et al., 2018). This raises the question whether the mechanisms of selection differ between these two phenomena and, if so, how. In the case of the conserved functional shifts in the microbiome due to the stress-response, perhaps this can be explained by a conserved physiological stress response in the host. In contrast, the more preserved succession of microbiota during early life development may be more dependent on the infant

immune system, which is known to be influenced by the maternal microbiome (Mueller et al., 2015).

7.2.3 Functional metagenomics analysis can provide more sensitivity and interpretability in microbiome-gut-brain axis studies.

Functional metagenomics analysis is an increasingly common feature of microbiome-gut-brain axis studies. We utilised functional metagenomics analysis next to taxonomical metagenomics analysis in several of the pieces of research discussed above. It's striking that the type of information we can glean from functional analysis seems to differ depending on the research. In some cases, like in chapter 3.3, documenting the colonization of *Blautia*, functional analysis can contribute to the interpretation of one's findings by confirming that alterations in a given bacterial taxon indeed would have functional consequences. In chapter 2.4, regarding the effect of prebiotics on chronic hypoxia, the functional analysis echoed what we saw in the microbiome analysis, being a strong effect of prebiotics and only a weak effect of hypoxia. Interestingly, we tend to see a strong effect in the capacity of the microbiome to metabolize substrates in studies where prebiotics are administered. In other cases, functional analysis is more sensitive than taxonomical level analysis. For instance, in chapter 2.3, the effects of polyphenols on the functional microbiome were more pronounced and numerous than on the taxonomical level. Another important example of an effect only visible on the functional level is the potential of the microbiome to metabolize levodopa (van Kessel et al., 2019). In chapter 6.3, which follows 24 participants on a diet high in unpasteurized dairy, we use the functional microbiome in a different manner. There, we show that after the course, the participants showed potential for a higher functional richness in regards to neuroactive functions.

In general, it is clear that functional analysis can represent a useful complementary analysis next to the more traditional taxonomical analysis. Another advantage to functional analysis is that it can make interpretation more straightforward. Especially in the case of 16S analysis, where we are usually constrained to the genus level, is

can be hard to defend that an entire genus is either “good” or “bad” for host health. On the other hand, functional analysis provides the researcher with molecular pathways that in many cases can lead to more defensible and concrete molecular mechanistic understanding. In the case where both microbiome and gut metabolomics measurements were taken, it would even be possible to integrate the two approaches using a framework like KEGG to confirm which molecular pathways are differentially affected.

7.2.4 There exists random drift in the microbiome over time, though intra-subject variance is smaller than inter-subject variance.

The microbiome can be seen as a dynamic ecosystem, in constant flux. Compositional stability and variability of the microbiome are investigated in several of the reports presented above. Most clearly in chapter 5.1, where volatility, the degree of change in the microbiome over time, is shown to be correlated to severity of the stress response in both mice and a healthy human cohort. In human cohorts, such as the cohort of students undergoing academic stress featured in chapter 5.1 for volatility measurements, but also the human cohorts from chapters 6.2, and 6.3, on probiotic administration in an obese cohort and the consumption of unpasteurized dairy, respectively, which both feature samples at multiple time points per participant, intra-subject variance of the microbiome over time was smaller than inter-subject variance. Indeed, other research groups report this as well (Johnson et al., 2019).

There are several important points to be made there. First, the fact that a previous composition of the microbiome holds some information on the next composition of the microbiome is an important confirmation that the microbiome is a consistent community with a drive towards homeostasis. Second, we can go further and say that this homeostasis is at least in part determined by function in relation to the host. Some interesting theoretical works has shown that different functions in a microbiome show different levels of robustness to perturbation, specifically, functions that were associated with a given biogeographical region were more robust in the microbiomes of that region (Eng and Borenstein, 2018). In many cases, the

reverse is also shown to be true. The microbiome compositions of healthy subjects are shown to be much more similar to each other than those of patients in numerous but not all microbiome-associated diseases, notably including Parkinson's Disease and Schizophrenia (Ma, 2020). This phenomenon is referred to as the Anna Karenina principle and has been applied to a wide array of academic fields. In these cases, the microbiome-associated disease may rather be modulated by a dysregulated microbiome, a lack of stability, rather than a certain stable pathogenic state like in the case of a disease like *Clostridium difficile* infection.

In the case of rodents, there have been reports of cage and litter effects in the microbiome. Indeed, as both rats and mice are known to be coprophagous, it stands to reason that the microbiomes of cage-mates would be converge towards each other. This is also reflected in the scientific literature (Ericsson et al., 2018; Miyoshi et al., 2018) and it is considered best practice to properly account for these effects during the experimental design. In humans, it has been reported that cohabitants have a more similar microbiome than average (Finnicum et al., 2019). The driving forces that determine in what way the microbiomes of cohabitants or cage-mates converge remains to be speculated on.

7.3 Identifying Strengths in the analysis of our studies

Limitations notwithstanding, there are several aspects that strengthened our understanding and yielded particularly informative or otherwise interesting results. It is worth discussing these so that future experiments can be tailored in such a way to ensure it can take advantage of these strengths.

7.3.1 Specific functional modules over a general functional analysis

As mentioned in the limitations, functional metagenomics analysis represents a valuable addition to taxonomical microbiome analysis, the two often complement each other. In several of our manuscripts, we went a step further and used the functional module framework. There are currently two types of functional modules, both introduced by the Flemish Raes group (Valles-Colomer et al., 2019). To reiterate,

first, there are Gut-Metabolic Modules (GMMs), which represent collections of the metabolic pathways and reactions that cover the genetic capacity of the microbiome to metabolise a given substrate. Next, there are Gut-Brain Modules (GBMs) which cover pathways and reactions that are known to synthesise or degrade neuroactive compounds, such as serotonin or GABA or histamine. There are many advantages to using these functional modules rather than a full non-curated list of all genes in a metagenome. First, functional modules represent a much shorter list than all genes or KEGG orthologues, making the results more practically feasible to interpret and reducing the risk of cherry-picking your favourite result from a long list of functions. Second, focusing on a shorter list of specific modules reduces the severity for post-hoc correction of the false discovery rate. This way, changes that are relevant to the researcher are more likely to remain detectable as significantly altered without being drowned out by other functions that would not have been relevant to the research question anyway. Third, the functional modules represent a convenient common ground between different studies. The type of database or sequencing technique used during analysis determines the exact annotation and naming of taxa and may even determine what functional databases the researcher has access to. However, as the functional modules represent a small list of pre-defined processes, it is trivial to compare them between studies. This makes it easier to compare and contrast ones findings with that of other groups with similar questions or manipulations, without necessitating a complete concordance in bioinformatics pipelines. Especially the ability to calculate functional modules in both 16S, like we did in chapters 2.3, 3.1, 5.1, and 6.3, as well as in whole genome shotgun sequencing, like we did in chapter 2.4, on prebiotics and hypoxia and the microbiome, greatly improves our ability to compare effects in the microbiome between these two approaches. Moving forward with functional modules, the ability to assess how many taxa have the capacity to perform certain function as well the degree of contribution of each of those taxa to a function seems like an excellent point to expand on. In chapter 3.3, we were able to use this approach to show that *Blautia* was the main contributor to the functions that were altered in the group receiving prebiotics after antibiotics perturbation of the microbiome. We were not able to find a difference in the number of taxa capable

of performing those functions in that study, but it would be interesting to find cases where this division of labour, so to speak, is indeed altered.

7.3.2 Volatility encapsulates the dynamic nature of the microbiome

It is well-known that the microbiome is a complex dynamic ecosystem that spans all kingdoms of life. It is known to be in constant flux, yet, this dynamic nature is rarely considered in the context of host health. Volatility was shown to be related to host state, not only in chapter 5.1, on volatility specifically, where it was linked to stress, but also in chapter 3.1, where aged mice receiving young donor FMT showed a lower volatility than those who received FMT from aged donors. We argue for the inclusion of volatility in all future research that permits it, in a way similar to how alpha-diversity is often considered. Further research on volatility itself should focus on the mechanisms determining what makes a microbiome volatile. In a longitudinal study following 34 participants over 17 consecutive days, the Knights group showed that stability of the microbiome was not related to stability of the diet, but that it was dependent on the diverseness of the diet (Johnson et al., 2019). This could mean that the host gut lumen metabolic environment regulates microbiome volatility.

Volatility in the context of the microbiome-gut-brain axis aside, incorporating multiple measurements from different time points has several advantages from different points of view. First, from an ecological standpoint, taking several observations per sample reveals information about stability and robustness of an ecosystem. Currently, we are not able to estimate whether a community, like a microbiome, would be stable just from a single metagenomics sample. Indeed, in chapter 5.1, on volatility, we were unable to pin down any features of the microbiome at baseline that may reveal anything about the volatility that microbiome would display over the course of the experiment. However, this information may be quite relevant to the researchers. Not only would a less stable, more volatile microbiome be reason to suspect negative health outcomes, perturbing a microbiome may increase permissivity for new taxa, potentially pathogens, to colonize it as seen in chapter 3.3, where antibiotics perturbation was

necessary for *Blautia* to colonize. *Blautia* is typically seen as a commensal genus, but it is hard to imagine that this is the only genus that can colonize the microbiome after a perturbation. Second, from a statistical standpoint, taking multiple measurements allows the researcher to use more sensitive statistical models and tests, potentially reducing the required n-number to be able to detect the alterations taking place. The most simple example of this would be that a paired t-test is more sensitive than an unpaired t-test. We used this to our advantage in chapters 3.1, on FMT and ageing and 5.1, on volatility, but the approach paid off especially well in the human studies in chapter 6 where we had multiple timepoints available; 6.2 and 6.3. It is unsurprising that human microbiomes show more unexplained variance than laboratory murine microbiomes in general, given the larger variance in genetics, diet and environment for humans just to name a few factors. Using the baseline microbiome, we were able to sidestep some of it, improving our power. Notably, The Knight group has recently released a tensor-based statistical model that takes intra-subject variability into account, improving sensitivity compared other common methods (Martino et al., 2020). Notably, while the manuscript does describe it as a way to take advantage of longitudinal data, the model is completely invariant to the order of the measurements and can also be used if the repeated measurement range from different microbiota in from the same host, further increasing possibilities.

7.4 Identifying Limitations of Microbiome Studies in the Microbiota-Gut-Brain Axis Field

There are several key limitations that should be considered when it comes to microbiome studies, including the ones discussed here. Some of these limitations could be seen as features of metagenomics sequencing. It should also be noted here that any metagenomic analysis that involves database-assisted annotation is dependent on experimental work. We simply could not perform high-throughput microbiome analysis without the massive amount of work performed in the wet lab.

7.4.1 There are inherent hidden biases in all metagenomic sequencing data

It is inherently problematic to compare microbiome between cohorts and, indeed, between plates within the same cohort. This phenomenon was thoroughly described and is invariant to 16S sequencing or whole genome shotgun metagenomics (McLaren et al., 2019). In a nutshell, it is not possible to infer abundance of a microbe or even the ratio between the abundance of two microbes in a single microbiome based on the amount of reads ascribed to said microbe. This is because hidden, often unknowable factors such as the ability of the microbial cell wall to withstand lysis or the resistance of the microbial genetic material to catalases will differentially affect the different microbes in a sample. Indeed, some of these biases are shown to be specific to a laboratory or even a sequencing plate, making it problematic to compare microbiome metagenomic sequencing data between batches or experiments. Fortunately, this does not impede our ability to investigate the microbiome outright. Within a batch, all biases are assumed to be equal. Furthermore, while the actual amounts of reads may differ based on these biases, the ratios of taxa can still be used to compare between cohorts. For example, if the proportion of, say, *Lactobacillus* in a sample increases based on the age of the host in a cohort, we would expect this to be the case in other cohorts as well, though the actual read count levels may vary to several orders of magnitude. Other metrics that don't rely on singular taxa or features, such as volatility, have also been shown to be robust to this hidden bias effect as is indeed the case in chapter 5.1, on volatility.

7.4.2 There are limitations specific to 16S amplicon sequencing

With the exception of chapter 2.4 on hypoxia and prebiotics in rats, all studies presented here feature 16S sequencing rather than whole genome shotgun metagenomics. While there are several advantages to 16S sequencing such as the lower price, faster processing time and the need for less biomaterial, the microbiome field is moving towards whole genome shotgun metagenomics. There are arguably three main limitations of 16S sequencing. Firstly, the 16S ribosomal RNA is virtually

exclusively found in prokaryotes, making the technique unsuitable to investigate the eukaryote and otherwise non-prokaryote section of the microbiome, which includes fungi, archaea, protists, some animalia like helminths and viruses like bacteriophage. Secondly, 16S sequencing is typically thought of as only being reliably up to the genus level, whereas whole genome shotgun metagenomics have been used to go to the strain level and even single nucleotide variants (Zeevi et al., 2019). This worse resolution can and does lead to the conflation of different strains within a genus. For instance, there have been instances of researchers using 16S sequencing to assess colonization efficiency of certain probiotics without the ability to actually detect these bacteria in this probiotic (Zmora et al., 2018). Thirdly, as 16S only picks up (part of the) the 16S sequence, the technique does not provide a direct way to investigate the functional capacity of the microbiome. Rather, inferential tools such as PICRUSt2 and Piphillin are necessary to take a best guess at the functional repertoire of the microbiome. Fortunately, both of these techniques perform reasonably well compared to whole genome shotgun sequencing, making this limitation generally not as limiting as the first two. In the case where a conclusion hinges on the presence or absence of a certain function, it is certainly recommended to verify the actual presence of a gene or functional pathway by sequencing it, be it by whole genome shotgun metagenomics or simply by targeted qPCR.

7.4.3 Many microbiome studies lack a longitudinal component

Longitudinal microbiome studies have several advantages over their single time point counterparts. As we discussed in chapter 5.1, on volatility, longitudinal studies enable us to investigate volatility, which has been shown to contain information on both the microbial ecosystem and on host health. Furthermore, as discussed in chapter 7.2.4, there is a large degree of intra-subject variance that can somewhat be accounted for by using a baseline measurement of each participant as its own control. Indeed, a framework to utilize multiple measurements per subject in order to reduce this intra-subject variance was recently proposed by another group (Martino et al., 2020).

Arguably similarly, one would be able to drastically reduce this variance as well by harnessing the longitudinal nature of a data set, simply employing a linear mixed effects model, with the random factor being the subject (donor). Because of the dynamic state of the microbiome, not taking baseline measurements forces the researches to assume that all microbiomes have a similar state at baseline. Fortunately, this is often a reasonable and justifiable assumption, but it is important to consider nonetheless. For instance, in the case of chapter 5.1, on volatility, the two cohorts of mice had a completely different microbiome at baseline. Not taking this into account would have likely led to a drastically different outcome. When linking microbiome data to host behaviour, cognition or mental health, there is an added difficulty in that many of the tests that are performed to assess these factors are subject to a learning effect, meaning that these tests themselves cannot necessarily be performed at all the time points one intends to take microbiome samples.

7.4.4 Many microbiome studies lack a functional component

In many cases when reporting on microbiome-gut-brain axis experiments, the researchers will speculate on the effects of significantly affected microbial taxa on the host. While this is not problematic on itself, it often makes more sense to rather look at the potential functional changes in the microbiome. In certain cases, it is completely justified to use both approaches side by side. For instance, in chapter 6.3, which focuses on a diet enriched in unpasteurized dairy, it makes sense to look at genera that are typically seen as lactic acid bacteria. Still, in the same research, we would not have been able to find an increase in neuroactive potential in the microbiome after the course without looking at the functional level. Several of the papers presented above would have benefited from functional analysis. For instance, consider chapters 2.1 and 2.2, which both deal with a dietary intervention targeting the microbiome, the first being a prebiotic intervention to rescue the effects of ageing and the second showing that dietary supplementation of omega-3 fatty acids and vitamin A rescues the effects of stress. It would have been interesting to investigate whether changes in the microbiome could have been explained based on the metabolic potential of said microbiome, or whether the observed

neuroprotective and anxiolytic effects could be linked to a change in the neuroactive potential of the microbiome. While 16S sequencing is not optimal for this type of analysis, inferential methods can still give a reasonable indication of what may be taking place.

7.5 Moving Forward

The microbiome-gut-brain axis field has undergone rapid development in the past decades and it will likely continue to do so for some time. New technologies and resources as well as a growing scientific knowledge will lead to a more mechanistic understanding of the microbiota-gut-brain axis in health and disease. Based on the research discussed above, we will discuss and in some cases recommend how to move the field forward.

7.5.1 Improving the bioinformatics

Because of the high-dimensional data nature of microbiome sequencing data, bioinformatics have played an important role in the microbiome-gut-brain axis field. In recent years, reproducibility has vastly improved, which many prominent journal strongly recommending researchers to publish their bioinformatics analysis scripts alongside their work. Yet, there are still some points that stand out to us in particular when it comes to moving the field forward. First, there is no consensus in which bioinformatics approach to take. Numerous tools, frameworks, pipelines and platforms have been proposed over the years. While this rapid growth in the amount of available resources should be seen as a boon in many ways, there is one major downside to them. Often, it is problematic to compare between microbiome studies because of differences in bioinformatics methodology. For instance, reference databases can evolve rapidly, some even yearly, sometimes reassigning entire clades such as was the case for *Lactobacillus* (Salveti et al., 2018). This difficulty to compare between studies makes it particularly problematic to perform meta-analyses. Often, reanalysing raw sequencing reads to is cumbersome and the quality of metadata is still insufficient. Besides the obvious solution of all using the same database, ways to

convert between databases, such is the case between many functional databases seems like the way forward. Another interesting approach could be meta-feature analysis with tools like the functional Gut-Brain Modules or Gut-Metabolic Modules. These tools are mostly agnostic to the upstream bioinformatics pipelines that were used, but still give outputs in the same format. Similar modules, be they related to function, taxonomy or something else, may enable us to converge on outcomes that are similar enough to compare without enforcing any one pipeline on researchers. In some cases like the use of the GreenGenes database, which was last updated in 2013, this type of reconciliation may still prove difficult. Second, microbiome metagenomics datasets are compositional in nature. This comes with a host of features not seen in classical datasets, such as an internal negative correlation between features within the data. In other words, if we know that one taxon takes up 10% of the population in one sample and 90% in the next sample, we already know as a result of this, that the rest of the taxa in the former sample encompass a lot more of the population than in the latter sample. The relative abundances are thus negatively correlated within the sample. Fortunately, compositional data analysis is a well-described field and there exist straightforward transformations, such as ALR, CLR and ILR, to essentially get rid of these effects and allow us to apply conventional statistical approaches (Aitchison, 1982; Aitchison et al., 2000). Recently, there have been some microbiome publications in high-impact journals that employ or even directly introduce adaptations of compositional data analysis effectively, thus improving the accuracy of their outcomes and improving the statistical approaches of the field as a whole (Johnson et al., 2019; Valles-Colomer et al., 2019; Martino et al., 2020).

7.5.2 Integrating ecological understanding

The microbiome-gut-brain axis field is a clear example of an interdisciplinary field, featuring aspects ranging ecology to molecular biology to gastroenterology to psychology, just to name a few. Notably, whereas many of these fields have other

areas of studies where they may overlap, it's safe to say that generally ecologists don't cross paths with the other fields as regularly. Microbe-microbe interactions are known to determine the behaviour of individual gut microbes and this is likely to also affect their neuroactive potential. There are several ecological subjects that we deem likely will be developed more deeply in terms of the microbiome. First, there is colonization. Indeed, during early life, the colonization of the microbiome is known to be important for normal development of the immune system and behaviour, like we also show in chapter 2.5, on the enduring effect of high fat and cafeteria diet, chapter 3.2, on the lingering effects of antibiotics during adolescence and chapter 3.4, on the lingering effect of caesarean section. There are indications the host allies a selective pressure on the microbiome to control this colonization (Ferretti et al., 2018). Furthermore, the microbiome has been shown to follow some patterns in how it recolonizes after antibiotics depletion (Chng et al., 2020). Understanding the factors determining healthy colonization, including that of psychobiotics, will undoubtedly prove beneficial to the microbiome-gut-brain axis field as a whole. Second, there are ecological guilds. Ecological guilds represent taxonomically unrelated but functionally related species that typically perform some sort of 'task' in the ecosystem, such as the degradation of a certain otherwise hard to process material (Simberloff and Dayan, 1991; Zhao et al., 2018). It is often of little consequence which members of a guild are present, but rather that there are some guild representation at all. In the microbiome, we are aware of two lines of research that try to elucidate the dynamics of guilds in the microbiome. First, Liping Zhao and colleagues have reported on the restoration of ecological guilds in patients with type 2 diabetes and argue for the experimental identification of guild members in order to efficiently screen for host health risks (Lam et al., 2018). Second, there is the study of trophic levels in the microbiome (Wang et al., 2019). In short, rather than microbiome being a chaotic collection of microbes that randomly metabolize based on what genes they may possess, the trophic levels model describes a hierarchy of sub-consortia of the microbiome, each feeding on metabolites produced by the lower layer and producing metabolites for the next layer. Interestingly, this phenomenon leads back to the first point of colonization as a trophic layer can only develop if the necessary lower trophic layer is already present in the microbiome

(Gralka et al., 2020). It seems likely that early colonizers such as members of *Lactobacillus* or *Bifidobacterium* could represent the base trophic level in both humans and rodents, given their importance in early life development and colonization of the microbiome, as also discussed in chapter 3.4, documenting the effects of caesarean section on the microbiome and neurobehaviour. Moving forward, it would be beneficial to develop a framework to identify or recognise ecological guilds in a fashion similar to the framework used to detect functional modules. Thirdly, there are enterotypes and the core microbiome, two concepts that are deeply related. It remains a point of discussion in the microbiome field whether enterotypes exist in the human population. In a nutshell, enterotypes can be thought of as stereotypical microbiomes, perhaps related to ones diet and lifestyle. In contrast, the core microbiome could arguably be thought of as the single viable, healthy enterotype, if there indeed is such a thing. Typically, in research arguing for the existence of enterotypes, three or four different ones are distinguished. Typically, at least one enterotype is characterised by its high relative abundance of *Bacteroides*, while another one has a high proportion of *Prevotella*, though the difficulties in comparing between studies and cohorts that was discussed in chapter 7.2.2 on commonalities between studies and chapter 7.4.1 in the limitations section make it problematic to make strong descriptive statements in this regard. In a seminal study, the Flemish group that also introduced functional modules showed that psychological quality of life scores are unevenly distributed between the four enterotypes they distinguished in their cohort of 1054 participants (Valles-Colomer et al., 2019). Going further, another study by a different group showed that certain intermediate states between two enterotypes are much less likely to occur than others, with metaphorical barriers preventing one enterotype to transition into a different enterotype, in addition to either enterotype being stable on itself (Levy et al., 2020). Thus, it seems likely that the enterotype framework holds scientific merit and furthermore that it holds predictive power when it comes to the microbiome-gut-brain axis. However, enterotypes are not considered when applying linear modelling approaches in the microbiome, which typically assume that all samples come from the same distribution. This stands in direct contrast with the concept of enterotypes. Looking forward, identifying enterotypes as part of microbiome

analysis, perhaps using some sort of reference tool or database, would enable bioinformaticians to take enterotypes into account in their statistical modelling approaches.

All models are wrong, but some are useful

George P.Box

7.5.3 Advancing the microbiome-gut-brain axis field

Throughout this text we have recommended numerous way in which to continue researching the microbiome-gut-brain axis field. Most notably, there is a lack of longitudinal studies. For instance, investigating microbiome stability and volatility in different types of stress or other psychiatric conditions such as major depressive disorder may lead to a deeper understanding of the mechanisms of gut-brain communication at play. Recently, more longitudinal studies have been published in the microbiome-gut-brain axis field. Psychotropic drugs were shown to affect the microbiome in a clinical population with anxiety and depressive disorder, including changes in neuroactive Gut-Brain Modules (Tomizawa et al., 2021). Another aspect of microbiome volatility that would be interesting to investigate is the interplay between volatility and other examples of stable changes, such as is the case in the circadian rhythm and the menstrual cycle. Other ways to characterize the microbiome, such as through metabolomics, should also be considered for volatility. We have inferentially shown that microbiome function is affected as part of volatility, but whether, and to what degree, the metabolic environment is affected still remains to be answered. Another important goal is the integration of microbiome datasets with other 'omics data, such as host transcriptomics or metabolomics. There are two main ways to approach this, one currently common than the other. First, there is naive integration. This involves simply correlating all features from the first data set to those of the other. Notably, the names of these features holds no weight in this analysis. Second, there is informed integration. This method is more rare and

typically relies on bioinformatics scripts rather than tools. Here, it matters which features are compared between datasets, drastically reducing possibility-space for correlations to occur and in turn increasing power. For example, metabolites from serum metabolomics may be compared to just the genes encoding for enzymes with said metabolites as known ligands. This approach is dependent on the quality of databases and was recently leveraged in a publication (Kim et al., 2020). Often, these methods are documented poorly or even unavailable altogether. In the study where we investigated the potential of FMT from young mice to reverse the effects of ageing in mice, I ended up developing my own method to integrate metabolomics and functional microbiome metagenomics using a biologically informed framework, by leveraging the KEGG framework. This method allowed me to specifically investigate whether relevant functions in the microbiome were altered based on the metabolites whose levels were found to be altered by ageing and rescued by FMT from young animals. Integrating data from different 'omics approaches can yield a clearer view of the metabolic pathways that play a role in microbiome-gut-brain communication, driving the field towards hypotheses that can lead to mechanistic understanding. Third, the effectiveness of the functional modules approach warrants an expansion of this type of framework. Rather than assessing the potential to metabolize neuroactive compounds, one could imagine a framework that assesses risk factors for mental health conditions. Furthermore, quantification of a microbiomes capacity to metabolize different psychobiotic drugs, its degree of antibiotic resistance and its permissivity to be colonized by new taxa, be they pathogenic or beneficial to the host come to mind as factors that would greatly improve interpretability of microbiome studies. Furthermore, as discussed in chapter 7.3.1, in the strengths section, an overlaying framework of modules that are invariant to upstream bioinformatics pipelines will enable researchers to reliably and compare outcomes between cohorts, also improving our ability to perform meta-analyses.

7.6 Conclusion

The microbiome is involved in regulating host health, including mental health. Furthermore, microbiome perturbation during critical windows of development can have lasting behavioural and neurophysiological consequences. The studies discussed here not only confirm this, but also build further upon previous studies investigating the microbiome-gut-brain axis. Furthermore, we have investigated and advanced our understanding of neglected features of the microbiome that we have shown to be informative in regard to the microbiome-gut-brain axis. Prime among these are volatility and functional modules. We have demonstrated how to measure these features and we have discussed how to leverage them in experiments. Additionally, we have argued for a stronger integration of ecological knowledge into our model of the gut microbiome, which we find to be essential in order to explain the results we find. Based on our findings, we have recommended new lines of questioning for future research. Future research incorporating volatility, functional markers and an ecological understanding from the design stage will enable us to move the field forward towards a deeper mechanistic understanding of microbiome-gut-brain communication. With this mechanistic understanding, we will be able to more effectively and safely develop therapies targeting the microbiome for mental health.

Chapter 8 References

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